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PATENT APPLICATION TRANSMITTAL LETTER

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To the Assistant Commissioner for Patents:

Transmitted herewith for filing under 35 U.S.C. 111 and 37 CFR 1.53 is the patent application of:

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entitled ***Patched Genes and Uses Related Thereto***

Enclosed are:

(X) 82 pages of written description, claims and abstract.
(X) 5 sheets of drawings.
( ) an assignment of the invention to _____.
( ) executed declaration of the inventors.
( ) a certified copy of a _____ application.
( ) a verified statement to establish small entity status under 37 CFR 1.9 and 1.27.
(X) other: unexecuted Declaration, Petition and Power of Attorney.

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Patched Genes and Uses Related Thereto

Related Applications

This application is a continuation-in-part of U.S.S.N. 08/656,055, which is a continuation-in-part of U.S.S.N. 08/540,406, which is a continuation-in-part of U.S.S.N. 08/317,745 (now abandoned). The specifications of each of these prior applications are incorporated herein by reference.

Background of the Invention

Segment polarity genes were originally discovered as mutations in flies that change the pattern of body segment structures. Mutations in these genes cause animals to develop changed patterns on the surfaces of body segments; the changes affecting the pattern along the head to tail axis. Among the genes in this class are *hedgehog,* which encodes a secreted protein (HH), and *patched,* which encodes a protein structurally similar to transporter proteins, having twelve transmembrane domains (*ptc*), with two conserved glycosylation signals.

The *hedgehog* gene of flies has at least three vertebrate relatives- *Sonic hedgehog (Shh); Indian hedgehog (Ihh), and Desert hedgehog (Dhh). Shh* is expressed in a group of cells, at the posterior of each developing limb bud, that have an important role in signaling polarity to the developing limb. The *Shh* protein product, SHH, is a critical trigger of posterior limb development, and is also involved in polarizing the neural tube and somites along the dorsal ventral axis. Based on genetic experiments in flies, *patched* and *hedgehog* have antagonistic effects in development. The *patched* gene product, *ptc*, is widely expressed in fetal and adult tissues, and plays an important role in regulation of development. *Ptc* downregulates transcription of itself, members of the transforming growth factor and *Wnt* gene families, and possibly other genes. Among other activities, HH upregulates expression of *patched* and other genes that are negatively regulated by *patched.*

It is of interest that many genes involved in the regulation of growth and control of cellular signaling are also involved in oncogenesis. Such genes may be oncogenes, which are typically upregulated in tumor cells, or tumor suppressor genes, which are down-regulated or absent in tumor cells. Malignancies may arise when a tumor suppressor is lost and/or an oncogene is inappropriately activated. Familial predisposition to cancer may occur when there is a mutation, such as loss of an allele encoding a suppressor gene, present in the germline DNA of an individual.

The most common form of cancer in the United States is basal cell carcinoma of the skin. While sporadic cases are very common, there are also familial syndromes, such as the basal cell nevus syndrome (BCNS). The familial syndrome has many features indicative of abnormal embryonic development, indicating that the mutated gene also plays an important role in development of the embryo. A loss of heterozygosity of chromosome 9q alleles in both familial and sporadic carcinomas suggests that a tumor suppressor gene is present in this region. The high incidence of skin cancer makes the identification of this putative tumor suppressor gene of great interest for diagnosis, therapy, and drug screening.

Relevant Literature

Descriptions of *patched,* by itself or its role with *hedgehog* may be found in Hooper and Scott (1989) Cell **59**-.751-765; and Nakano *et al.* (1989) Nature **341** -.508-513. Both of these references also describe the sequence for *Drosophila patched.* Discussions of the role of *hedgehog* include Riddle *et al.* (1993) Cell 75-.1401-1416-, Echelard *et al.* (1993) Cell **75**:1417-1430- Krauss *et al.* (1993) Cell **75**:1431-1444 (1993); Tabata and Kornberg (1994) 76:89-102; Heemskerk and DiNardo (1994) Cell **76**:449-460; and Roelink *et al.* (1994) Cell **76**:-761-775.

Mapping of deleted regions on chromosome 9 in skin cancers is described in Habuchi *et al.* (1995) Oncogene **11**: 1 671-1674, Quinn *et al.* (1 994) Genes Chromosome Cancer **11**:222-225; Quinn *et al.* (1994) J. Invest. Dermatol. 102:300-303; and Wicking *et al.* (1994) Genomics **22**:505-51 1.

Gorlin (1987) Medicine **66**:98-113 reviews nevoid basal cell carcinoma syndrome. The syndrome shows autosomal dominant inheritance with probably complete penetrance. About 60% of the cases represent new mutations. Developmental abnormalities found with this syndrome include rib and craniofacial abnormalities, polydactyly, syndactyly and spina bifida. Tumors found with the syndrome include basal cell carcinomas, fibromas of the ovaries and heart, cysts of the skin, jaws and mesentery, meningiomas and medulloblastomas.

Summary of the Invention

Isolated nucleotide compositions and sequences are provided for *patched (ptc)* genes, including mammalian, e.g. human and mouse, and invertebrate homologs. Decreased expression of *ptc* is associated with the occurrence of human cancers, particularly basal cell carcinomas and other tumors of epithelial tissues such as the skin. The cancers may be familial, having as a component of risk a germline mutation in the gene, or may be sporadic. *Ptc*, and its antagonist *hedgehog,* are useful in creating transgenic animal models for these human cancers. The *ptc* nucleic acid compositions find use in identifying homologous or

related genes; in producing compositions that modulate the expression or function of its encoded protein, *ptc*; for gene therapy; mapping functional regions of the protein- and in studying associated physiological pathways. In addition, modulation of the gene activity *in vivo* is used for prophylactic and therapeutic purposes, such as treatment of cancer, identification of cell type based on expression, and the like. *Ptc*, anti-*ptc* antibodies and *ptc* nucleic acid sequences are useful as diagnostics for a genetic predisposition to cancer or developmental abnormality syndromes, and to identify specific cancers having mutations in this gene.

Brief Description of the Drawings

Fig. 1 is a graph having a restriction map of about 10 kbp of the 5' region upstream from the initiation codon of *Drosophila patched* gene and bar graphs of constructs of truncated portions of the 5' region joined to fl-galactosidase, where the constructs are introduced into fly cell lines for the production of embryos. The expression of fl-gal in the embryos is indicated in the right-hand table during early and late development of the embryo. The greater the number of +'s, the more intense the staining.

Fig. 2 shows a summary of mutations found in the human *patched* gene locus that are associated with basal cell nevus syndrome. Mutation (1) is found in sporadic basal cell carcinoma, and is a C to T transition in exon 3 at nucleotide 523 of the coding sequence, changing Leu 175 to Phe in the first extracellular loop. Mutations 2-4 are found in hereditary basal carcinoma nevus syndrome. (2) is an insertion of 9 bp at nucleotide 2445, resulting in the insertion of an additional 3 amino acids after amino acid 815. (3) is a deletion of 11 bp, which removes nt 2442-2452 from the coding sequence. The resulting frameshift truncates the open reading frame after amino acid 813, 'just after the seventh transmembrane domain. (4) is a G to C alteration that changes two conserved nucleotides of the 3' splice site adjacent to exon 10, creating a non-functional splice site that truncates the protein after amino acid 449, in the second transmembrane region.

Fig. 3 (panels A-B) illustrates the generation of *ptc* mutations. (**A**) The *ptc* mutant allele was generated by homologous recombination between the KO1 targeting vector and *ptc*. External probe A detected a 3' EcoRV polymorphism on blots and probe B detected a 5' SacI polymorphism. Exons are numbered. (**B**) Transmission of the ptc^{KO1} allele through the germline was confirmed by Southern blot (upper panel) and a PCR genotyping assay (lower panel). PCR primers are indicated as arrows in **A**. Because the homozygous mutant embryos were being resorbed, there was much less yolk sac DNA in the -/- lanes.

Fig. 4 (panels A-G) illustrate the germ layer-specific derepression of Hh target genes in $ptc^{-/-}$ embryos. (**A, B**) Lateral views of E8.25 wild-type (**A**) and $ptc^{-/-}$ (**B**) embryos. The

headfolds are overgrown in the mutant (white arrows) and the heart is not properly formed (red arrows). (**C**) Lateral views of E8.75 *ptc*$^{+/-}$ (left) and *ptc*$^{-/-}$ (right) embryos stained with X-gal *(28)* (**D, E, F, G**) Transverse sections through E8.75 *ptc*$^{+/-}$ (**D, F**) and *ptc*$^{-/-}$ (**E, G**) embryos stained with X-gal (**D, E**) or hybridized with a digoxigenin labeled *Gli* probe *(29)* (**F, G**). Both *lacZ* and *Gli* were derepressed in the ectoderm and mesoderm but not in the endoderm (arrows). In **A** and **B**, anterior is to the left and dorsal is up. In **C**, anterior is up and dorsal is to the right. In **D** to **G**, dorsal is up.

Fig. 5 (panels A-L) illustrate ventralization of the neural tube in *ptc*$^{-/-}$ embryos. (**A**) Lateral view of E8.5 wild-type (left) and *ptc*$^{-/-}$ (right) embryos hybridized with a *HNF3b* probe. Expression is expanded dorsally in the mutant. (**B, C**) Transverse sections through the hindbrain of E8.5 wild-type (**B**) and *ptc*$^{-/-}$ (**C**) embryos hybridized with ^{35}S-labeled *Shh* probe *(8)*. *Shh* is expressed in the floor plate (fp) and notochord (nc) of the wild-type embryo, and is greatly expanded in the *ptc* mutant . g = gut (**D, E**) Hematoxylin and eosin stained transverse sections through the hindbrain of wild-type (**D**) and *ptc*$^{-/-}$ (**E**) E8.5 embryos. Bottle-shaped cells with basal nuclei are indicated by arrows. (**F, G**) Transverse sections through E8.5 *ptc*$^{+/-}$ (**F**) and *ptc*$^{-/-}$ (**G**) embryos hybridized with *Pax6* probe show loss of expression from the *ptc* mutant. (**H**) Dorsal view of E8.25-E8.5 embryos hybridized with *Pax3* probe. Because of the kinking in the neural tube, the *ptc*$^{-/-}$ embryo is curled on itself. Weak *Pax3* expression is seen in the posterior dorsal neural tube of the *ptc*$^{-/-}$ embryo (bottom, arrow). (**I, J**) Transverse sections through E8.5 wild-type (**I**) and *ptc*$^{-/-}$ (**J**) embryos hybridized with *Pax3* probe. *Pax3* is expressed in the dorsal neural tube (nt) and dermamyotome (dm) in the wild-type, but is only present in a small dorsal domain of the mutant neural tube. s = somite (**K, L**) Lateral views of E9 wild type (**K**) and E8.5 *ptc*$^{-/-}$ (**L**) embryos hybridized with *erb-b3* probe. Staining is seen in migrating neural crest in the head and somites of wild type but not mutant embryos (red arrows). Weak staining in the head, heart and gut (black arrows) is background or non-neural crest related. (**M**) Lateral view of wild type (top) and *ptc*$^{-/-}$ (bottom) embryos hybridized with *Nkx2.1* probe. The body of the mutant is twisted. *Nkx2.1* expression is limited to the anterior, but is expanded dorsally in the mutant. (**N**) Lateral view of E8.5 *ptc*$^{+/-}$ (left) and *ptc*$^{-/-}$ (right) embryos hybridized with *hoxb1* probe. Loss of expression in rhombomere four is indicated by the asterisks. In all transverse sections, dorsal is up. In **A**, **K**, **L** and **N**, anterior is up and dorsal is to the right. In **H** and **M**, anterior is to the left.

Fig. 6 (panels A-F) depict keletal abnormalities and medulloblastomas in *ptc*$^{+/-}$ mice (**A**) Alcian blue and Alizarin red stained hindlimb from a *ptc*$^{+/-}$ mouse *(30)*. The preaxial digit is duplicated (arrows). (**B, C**) Dorsal views of brains from wild-type (**B**) and *ptc*$^{+/-}$ (**C**) mice. Anterior is up. In the posterior wild-type brain, the colliculi (col) are present as distinct bumps between the cortex (cor) and cerebellum (ce). In the *ptc*$^{+/-}$ mouse, a massive medulloblastoma (mb, outlined in red) grew over the colliculi and normal

cerebellum, which can no longer be seen. The olfactory bulbs were removed. (**D, E**) Hematoxylin and eosin stained section through human (**D**) and mouse (**E**) medulloblastomas. The tumor cells are small with dark, carrot-shaped nuclei (arrows) and form nodules with no apparent orientation. (**F**) Synaptophysin immunoreactivity in a mouse medulloblastoma *(26)*. Synaptophysin staining (brown) is seen in some processes (arrows). Nuclei are purple.

Fig. 7 (panels A-G) illustrate derepression of *ptc* and *Gli* expression in medulloblastomas from $ptc^{+/-}$ mice. (**A to C**) Semi-adjacent sections through a tumor in the cerebellum of a $ptc^{+/-}$ mouse hybridized with ^{35}S labeled probes to *ptc* (**A**), *Gli* (**B**) and *Shh* (**C**). *ptc* and *Gli* transcripts are abundant in the tumors (asterisks) compared to nearby cerebellar tissue (arrows). No *Shh* was detected in the tumor. (**D**) $ptc^{+/-}$ cerebellum (ce) and tumor (mb) stained with X-gal *(28)*. Anterior is to the left. Derepression of *ptc* expression in the medulloblastoma is reflected in the high level of X-gal staining. (**E**) Surface staining in (arrows) regions of $ptc^{+/-}$ cerebellum contrast with absence of b-galactosidase activity in most folia (asterisk). (**F**) Sagittal section through cerebellum in **E**. X-gal staining nuclei (arrow) accumulated superficial to the molecular layer (ml), where stained nuclei are not normally seen. In unaffected regions of the cerebellum, X-gal staining was seen in scattered cells of the molecular layer (ml), strongly in the Purkinje cell layer (pcl) and weakly in the granule cell layer (gl). (**G**) *ptc* expression was examined in total RNA (15 mg) from wild-type (WT) and $ptc^{+/-}$ (+/-) cerebellums using a probe (M2-2) *(6)* that detects exons downstream of the *lacZ* and *neo* insertions. Actin mRNA was used as an RNA loading control. The $ptc^{+/-}$ mice had~50% decrease in *ptc* transcripts.

Database References for Nucleotide and Amino Acid Sequences

The sequence for the *D. melanogaster patched* gene has the Genbank accession number M28418. The sequence for the mouse *patched* gene has the Genbank accession number lt30589-V46155. The sequence for the human *patched* gene has the Genbank accession number U59464.

Detailed Description of the Invention

Vertebrate and invertebrate *patched (ptc)* gene compositions and methods for their isolation are provided. Of particular interest are mammalian *ptc* genes, such as the human and mouse homologs described in the appended examples. The *ptc* gene, in mammals, is a tumor suppressor and developmental regulator. Certain human cancers, e.g. basal cell carcinoma, transitional cell carcinoma of the bladder, meningiomas, medulloblastomas, etc., can be characterized by *ptc* loss-of-function, such as that resulting from oncogenic mutations at the *ptc* locus, or other loss-of-function mutations which decrease *ptc* activity in the cell. As

described below, we have observed somatic mutations in the *ptc* gene in a variety of sporadic cancers. For instance, the basal cell nevus syndrome (BCNS), an inherited disorder, is associated with germline mutations in *ptc*. Some patients with basal cell nevus syndrome (BCNS) have germ line mutations in *ptc*, and are at increased risk for developmental defects such as spina bifida and craniofacial abnormalities, basal cell carcinoma (BCC) of the skin, and brain tumors. Mutations to *ptc* genes are also observed to occur in sporadic BCCs, which generally have both copies of *ptc* inactivated.

The term "loss-of-function" is art recognized and, with respect to a *patched* gene or gene product refers to mutations in a *patched* gene which ultimately decrease or otherwise inhibit the ability of a cell to transduce *patched*-mediated signals, e.g., the cells may lose responsiveness to *hedgehog* induction. For example, a loss-of-function mutation to a *patched* gene may be a point mutation, deletion or insertion of sequences in the coding sequence, intron sequence or 5' or 3' flanking sequences of the gene so as to, for example, (i) alter (e.g., decrease) the level *patched* expression, (ii) alter exon-splicing patterns, (iii) alter the ability of the encoded *patched* protein to interact with extracellular or intracellular proteins (such as *hedgehog*), or (iv) alter (decrease) the stability of the encoded *patched* protein.

The term "aberrant modification" is art recognized and, with respect to a *patched* gene, refers to a a non-wildtype mutation or other alteration to the gene, e.g., which results in full or partial loss-of-function of the *patched* protein or expression of the *patched* gene.

Such mutations affecting *ptc* activity have also been associated with other human cancers, including carcinomas, adenocarcinomas, sarcomas and the like. Decreased *ptc* activity is also associated with inherited developmental abnormalities, e.g. rib and craniofacial abnormalities, polydactyly, syndactyly and spina bifida.

The art-recognized term "predisposing mutation", as it pertains to *patched* genes, refers to mutations to the *patched* gene which result in loss-of-function.

The term "genetic predisposition" is art recognized, and refers to a genotype of an animal which predisposes the animal to developing a certain pathological conditions with a frequency (probability) greater than the average for the overall population of that animal, taking into account, as appropriate, age, sex or other related physical or medical condition(s).

The *ptc* genes and fragments thereof, encoded protein, and anti-*ptc* antibodies are useful in the identification of individuals predisposed to development of a variety of cancers and developmental abnormalities, and in characterizing the phenotype of various tumors or other proliferative or degenerative disorders that are associated with this gene, e.g., for diagnostic and/or prognostic benefit. The characterization is useful for prenatal screening; and in determining the phenotype of a proliferative disorder, e.g. for determining a course of treatment of the patient. Tumors may be typed or staged as to the *ptc* status, e.g. by detection

of mutated sequences, antibody detection of abnormal protein products, and functional assays for altered *ptc* activity.

The terms "developmental disorder" and "develpmental abnormality" are art recognized, and refer to abberant development of a cell, tissue or organ, e.g., in size, symmetry or functional performance, which abnormality may or may not be untowardly manifest.

The term "proliferative disorder" is art recognized and refers to a disorder affecting an animal in a manner which is marked by abberant, or otherwise unwanted, proliferation of a subset of cells of an animal. Cancers are proliferative disorders.

The encoded *ptc* protein is also useful in drug screening for compositions that mimic *ptc* activity or expression, including altered forms of *ptc* protein, particularly with respect to *ptc* function as a tumor suppressor in oncogenesis.

The human and mouse *ptc* gene sequences and isolated nucleic acid compositions are provided in the appended examples. In identifying the mouse and human *patched* genes, cross-hybridization of DNA and amplification primers were employed to move through the evolutionary tree from the known *Drosophila ptc* sequence, identifying a number of invertebrate homologs.

The human *patched* gene has been mapped to human chromosome band 9q22.3, and lies between the polymorphic markers D9S196 and D9S287 (a detailed map of human genome markers may be found in Dib *et al.* (1996) Nature 280:152- http://www.genethon.fr).

As will be understood by those skilled in the art, the method of the present invention can be carried out using any of a large number of assay techniques for detecting alterations in *ptc* genes and/or *ptc* protein function. For instance, individuals are screened by analyzing their DNA or RNA for the presence of a predisposing oncogenic or developmental mutation, as compared to a normal sequence. An exemplary "normal" sequence of *patched* is provided in SEQ ID NO:19 (human). Specific mutations of interest include any mutation that leads to oncogenesis or developmental abnormalities, including insertions, substitutions and deletions in the coding region sequence, in the introns (e.g., that affect splicing), in the transcriptional regulatory sequences (such as promoter or enhancer sequences) that affect the activity and expression of the protein.

In general, the subject method can be characterized as including a step of detecting, in a sample of cells from a patient, the presence or absence of *ptc* expression (at the protein or mRNA transcript level), mutations to the *ptc* gene (coding or non-coding sequence) and/or the functional activity of *ptc* in the sample of cells (such as induction of Gli or the like). Moreover, the subject method can be used to assess the phenotype of cells which are known

to be transformed, the phenotype results being useful in planning a particular therapeutic regimen.

To illustrate, nucleic acid samples are obtained from a patient having, or suspected as being at risk for developing, a tumor or developmental abnormality which may be associated with *ptc*. The nucleic acid is analyzed for the presence of a predisposing mutation in the *ptc* gene. The presence of a mutated *ptc* sequence that affects the level of expression of the gene, stability of the gene product, and/or signal transduction activity of *ptc* confers an increased susceptibility to a proliferative or developmental disorder. Thus, the level of expression of *ptc* can be used predictively to evaluate whether a sample of cells contains cells which are, or are predisposed towards becoming, transformed.

Diagnostic/prognostic screening of tissue/cell samples for tumors or developmental abnormalities may also be based on the functional or antigenic characteristics of the protein. Immunoassays designed to detect the normal or abnormal *ptc* protein may be used in screening. Where many diverse mutations lead to a particular disease phenotype, functional protein assays have proven to be effective screening tools. Such assays may be based on detecting changes in the transcriptional regulation mediated by *ptc*, or may directly detect *ptc* activities such as *hedgehog* binding, transporter activity or the like, or may involve antibody localization of *patched* in cells.

Inheritance of BCNS is autosomal dominant, although many cases are the result of new mutations. Diagnosis of BCNS is performed by protein, DNA sequence or hybridization analysis of any convenient sample from a patient, e.g. biopsy material, blood sample, scrapings from cheek, etc. A typical patient genotype will have a predisposing mutation on at least one chromosome. In tumors and at least sometimes developmentally affected tissues, loss of heterozygosity at the *ptc* locus leads to aberrant cell and tissue behavior. When the normal copy of *ptc* is lost, leaving only the reduced function mutant copy, abnormal cell growth and reduced cell layer adhesion is the result. Examples of specific *ptc* mutations in BCNS patients are a 9 bp insertion at nt 2445 of the coding sequence- and an 11 bp deletion of nt 2441 to 2452 of the coding sequence. These result in insertions or deletions in the region of the seventh transmembrane domain.

Prenatal diagnosis of BCNS may be performed, particularly where there is a family history of the disease, e.g. an affected parent or sibling. It is desirable, although not required, in such cases to determine the specific predisposing mutation present in affected family members. A sample of fetal DNA, such as an amniocentesis sample, fetal nucleated or white blood cells isolated from maternal blood, chorionic villus sample, etc. is analyzed for the presence of the predisposing mutation. Alternatively, a protein based assay, e.g. functional assay or immunoassay, is performed on fetal cells known to express *ptc*.

Sporadic tumors associated with loss of *ptc* function include a number of carcinomas and other transformed cells known to have deletions in the region of chromosome 9q22, e.g. basal cell carcinomas, transitional bladder cell carcinoma, meningiomas, medullomas, fibromas of the heart and ovary, and carcinomas of the lung, ovary, kidney and esophagus. Characterization of sporadic tumors will generally require analysis of tumor cell DNA, conveniently with a biopsy sample. A wide range of mutations are found in sporadic cases, up to and including deletion of the entire long arm of chromosome 9. Oncogenic mutations may delete one or more exons, e.g. 8 and 9, may affect the amino acid sequence such as of the extracellular loops or transmembrane domains, may cause truncation of the protein by introducing a frameshift or stop codon, etc. Specific examples of oncogenic mutations include a C to T transition at nt 523 and deletions encompassing exon 9. C to T transitions are characteristic of ultraviolet mutagenesis, as expected with cases of skin cancer.

Biochemical studies may be performed to determine whether a candidate sequence variation in the *ptc* coding region or control regions is oncogenic. For example, a change in the promoter or enhancer sequence that downregulates expression of *patched* may result in predisposition to cancer. Expression levels of a candidate variant allele are compared to expression levels of the normal allele by various methods known in the art. Methods for determining promoter or enhancer strength include quantitation of the expressed mRNA or *ptc* protein; insertion of the variant control element into a vector with a reporter gene such as β-galactosidase, chloramphenical acetyltransferase, etc. that provides for convenient quantitation- and the like. Nuclear run-off assays are anpther convenient means for measuring promoter/enhancer activity. The activity of the encoded *ptc* protein may be determined by comparison with the wild-type protein, e.g. by detection of transcriptional regulation of TGF or *Wnt* family genes, Gli genes, *ptc* itself, or reporter gene fusions involving transcriptional regulatory sequences of these target genes.

The term "*patched*-dependent gene", or "a gene which is regulated in a *patched*-dependent manner", refers to genes, such as Gli or *patched*, etc, whose level of expression is regulated at least in part by the presence of a *patched* protein in the cell, e.g., can be controlled by *patched*-dependent intracellular signals.

A human *patched* gene (SEQ ID NO:18) has a 4.5 kb open reading frame encoding a protein of 1447 amino acids. Including coding and noncoding sequences, it is about 89% identical at the nucleotide level to the mouse *patched* gene (SEQ ID NO:9). A mouse *patched* gene (SEQ ID NO:9) encodes a protein (SEO ID NO:10) that has about 38% identical amino acids to *Drosophila ptc* (SEQ ID NO:6), over about 1,200 amino acids. The butterfly homolog (SEQ ID NO:4) is 1,300 amino acids long and overall has a 50% amino acid identity to fly *ptc* (SEQ ID NO:6). A 267 bp exon from the beetle patched gene encodes an

89 amino acid protein fragment, which was found to be 44% and 51% identical to the corresponding regions of fly and butterfly *ptc* respectively.

The DNA sequence encoding *ptc* may be cDNA, RNA, genomic DNA or synthetic, an includes fragments of the full-length coding sequence. The term *"patched* gene" shall be intended to mean the open reading frame encoding specific *ptc* polypeptides, as well as, as appropriate, adjacent intronic sequences and 5' and 3' non-coding nucleotide sequences involved in the regulation of expression, up to about 1 kb beyond the coding region, in either direction. The gene may be introduced into an appropriate vector for extrachromosomal maintenance or for integration into the host.

The term "cDNA" as used herein is intended to include all nucleic acids that share the arrangement of sequence elements found in native mature mRNA species, where sequence elements are exons, 3' and 5' non-coding regions. Normally mRNA species have contiguous exons, with the intervening introns deleted, to create a continuous open reading frame encoding *ptc*.

The genomic *ptc* sequence has a non-contiguous open reading frame, where introns interrupt the coding regions. A genomic sequence of interest comprises the nucleic acid present between the initiation codon and the stop codon, as defined in the listed sequences, including all of the introns that are normally present in a native chromosome. It may further include the 3' and 5' untranslated regions found in the mature mRNA. It may further include specific transcriptional and translational regulatory sequences, such as promoters, enhancers, etc., including about 1 kb of flanking genomic DNA at either the 5' or 3' end of the coding region. The genomic DNA may be isolated as a fragment of 50 kbp or smaller; and substantially free of flanking chromosomal sequence.

The nucleic acid compositions of the subject invention encode all or a part of the subject polypeptides. Fragments may be obtained of the DNA sequence by chemically synthesizing oligonucleotides in accordance with conventional methods, by restriction enzyme digestion, by PCR amplification, etc. For the most part, DNA fragments will be of at least 15 nt, usually at least 18 nt, more usually at least about 50 nt. Such small DNA fragments are useful as primers for PCR, hybridization screening, etc. Larger DNA fragments, i.e. greater than 100 nt are useful for production of the encoded polypeptide. For use in amplification reactions, such as PCR, a pair of primers will be used. The exact composition of the primer sequences is not critical to the invention, but for most applications the primers will hybridize to the subject sequence under stringent conditions, as known in the art. It is preferable to chose a pair of primers that will generate an amplification product of at least about 50 nt, preferably at least about 100 nt. Algorithms for the selection of primer sequences are generally known, and are available in commercial software packages.

Amplification primers hybridize to complementary strands of DNA, and will prime towards each other.

The *ptc* genes are isolated and obtained in substantial purity, generally as other than an intact mammalian chromosome. Usually, the DNA will be obtained substantially free of other nucleic acid sequences that do not include a *ptc* sequence or fragment thereof, generally being at least about 50%, usually at least about 90% pure and are typically "recombinant", i.e. flanked by one or more nucleotides with which it is not normally associated on a naturally occurring chromosome.

The DNA sequences are used in a variety of ways. They may be used as probes for identifying other patched genes. Mammalian homologs have substantial sequence similarity to the subject sequences, i.e. at least 75%, usually at least 90%, more usually at least 95% sequence identity with the nucleotide sequence of the subject DNA sequence. Sequence similarity is calculated based on a reference sequence, which may be a subset of a larger sequence, such as a conserved motif, coding region, flanking region, etc. A reference sequence will usually be at least about 18 nt long, more usually at least about 30 nt long, and may extend to the complete sequence that is being compared. Algorithms for sequence analysis are known in the art, such as BLAST, described in Altschul *et al.* (1990) J Mol Biol 215:403-10.

Nucleic acids having sequence similarity are detected by hybridization under low stringency conditions, for example, at 50 C and 10xSSC (0-9 M saline/0.09 M sodium citrate) and remain bound when subjected to washing at 55°C in 1xSSC. By using probes, particularly labeled probes of DNA sequences, one can isolate homologous or related genes. The source of homologous genes may be any mammalian species, e.g. primate species, particularly human- murines, such as rats and mice, canines, felines, bovines, ovines, equines, etc.

The DNA may also be used to identify expression of the gene in a biological specimen. The manner in which one probes cells for the presence of particular nucleotide sequences, as genomic DNA or RNA, is well-established in the literature and does not require elaboration here. Conveniently, a biological specimen is used as a source of mRNA. The mRNA may be amplified by RT-PCR, using reverse transcriptase to form a complementary DNA strand, followed by polymerase chain reaction amplification using primers specific for the subject DNA sequences. Alternatively, the mRNA sample is separated by gel electrophoresis, transferred to a suitable support, e.g., nitrocellulose and then probed with a fragment of the subject DNA as a probe. Other techniques may also find use. Detection of mRNA having the subject sequence is indicative of *patched* gene expression in the sample.

The subject nucleic acid sequences may be modified for a number of purposes, particularly where they will be used intracellularly, for example, by being joined to a nucleic acid cleaving agent, e.g. a chelated metal ion, such as iron or chromium for cleavage of the

gene; as an antisense sequence, or the like. Modifications may include replacing oxygen of the phosphate esters with sulfur or nitrogen, replacing the phosphate with phosphoramide, etc.

A number of methods are available for analyzing genomic DNA sequences. Where large amounts of DNA are available, the genomic DNA is used directly. Alternatively, the region of interest is cloned into a suitable vector and grown in sufficient quantity for analysis, or amplified by conventional techniques, such as the polymerase chain reaction (PCR). The use of the polymerase chain reaction is described in Saiki, *et al.* (1985) Science 239:487, and a review of current techniques may be found in Sambrook, *et al.* Molecular Cloning: A Laboratory Manual, CSH Press 1989, pp.14.2-14.33.

A detectable label may be included in the amplification reaction. Suitable labels include fluorochromes, e.g. fluorescein isothiocyanate (FITC), rhodamine, Texas Red, phycoerythrin, allophycocyanin, 6-carboxyfluorescein (6-FAM), 2',7'-dimethoxy-4',5'-dichloro-6-carboxyfluorescein (JOE), 6-carboxy-Xrhodamine (ROX), 6-carboxy-2',4',7',4,7-hexachlorofluorescein (HEX), 5-carboxyfluorescein (5-FAM) or N,N,N',N'-tetramethyl-6-carboxyrhodamine (TAMRA), radioactive labels, e.g. **32P, 35S,** 3H; etc. The label may be a two stage system, where the amplified DNA is conjugated to biotin, haptens, etc. having a high affinity binding partner, e.g. avidin, specific antibodies, etc., where the binding partner is conjugated to a detectable label. The label may be conjugated to one or both of the primers. Alternatively, the pool of nucleotides used in the amplification is labeled, so as to incorporate the label Into the amplification product.

The amplified or cloned fragment may be sequenced by dideoxy or other methods, and the sequence of bases compared to the normal *ptc* sequence. Hybridization with the variant sequence may also be used to determine its presence, by Southern blots, dot blots, etc. Single strand conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), and heteroduplex analysis in gel matrices are used to detect conformational changes created by DNA sequence variation as alterations in electrophoretic mobility. The hybridization pattern of a control and variant sequence to an array of oligonucleotide probes immobilized on a solid support, as described in WO 95/11995, may also be used as a means of detecting the presence of variant sequences. Alternatively, where a predisposing mutation creates or destroys a recognition site for a restriction endonuclease, the fragment is digested with that endonuclease, and the products size fractionated to determine whether the fragment was digested. Fractionation is performed by gel electrophoresis, particularly acrylamide or agarose gels.

In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers

which specifically hybridize to a *ptc* gene under conditions such that hybridization and amplification of the *ptc* gene (if present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In yet another exemplary embodiment, aberrant methylation patterns of a *ptc* gene can be detected by digesting genomic DNA from a patient sample with one or more restriction endonucleases that are sensitive to methylation and for which recognition sites exist in the *ptc* gene (including in the flanking and intronic sequences). See, for example, Buiting et al., (1994) Human Mol Genet 3:893-895. Digested DNA is separated by gel electrophoresis, and hybridized with probes derived from, for example, genomic or cDNA sequences. The methylation status of the *ptc* gene can be determined by comparison of the restriction pattern generated from the sample DNA with that for a standard of known methylation.

In still another embodiment, a diagnostic assay is provided which detects the ability of a *ptc* gene product, e.g., recombinantly expressed from a gene isolated from a biopsied cell, to bind to other proteins, e.g., upstream (*hedgehog*) or downstream of *ptc*. For instance, it will be desirable to detect *ptc* mutants which bind with lower binding affinity for *hedgehog* proteins. Such mutants may arise, for example, from fine mutations, e.g., point mutants, which may be impractical to detect by the diagnostic DNA sequencing techniques or by the immunoassays described above. The present invention accordingly further contemplates diagnostic screening assays which generally comprise cloning one or more *ptc* genes from the sample cells, and expressing the cloned genes under conditions which permit detection of an interaction between that recombinant gene product and a *ptc*-binding protein, e.g., a *hedgehog* protein. As will be apparent from the description of the various drug screening assays set forth below, a wide variety of techniques can be used to determine the ability of a *ptc* protein to bind to other cellular components.

The subject nucleic acids can be used to generate transgenic animals or site specific gene modifications in cell lines. Transgenic animals may be made through homologous recombination, where the normal patched locus is altered. Alternatively, a nucleic acid construct is randomly integrated into the genome, Vectors for stable integration include plasmids, retroviruses and other animal viruses, YACS, and the like.

The modified cells or animals are useful in the study of *patched* function and regulation. For example, a series of small deletions and/or substitutions may be made in the *patched* gene to determine the role of different exons in oncogenesis, signal transduction, etc. Of particular interest are transgenic animal models for carcinomas of the skin, where expression of *ptc* is specifically reduced or absent in skin cells. An alternative approach to transgenic models for this disease are those where one of the mammalian *hedgehog* genes, *e.g. Shh, Ihh, Dhh,* are upregulated in skin cells, or in other cell types. For models of skin

abnormalities, one may use a skin-specific promoter to drive expression of the transgene, or other inducible promoter that can be regulated in the animal model. Such promoters include keratin gene promoters. Specific constructs of interest include anti-sense *ptc*, which will block *ptc* expression, expression of dominant negative *ptc* mutations, and over-expression of HH genes. A detectable marker, such as *lacZ* may be introduced into the *patched* locus, where upregulation of patched expression will result in an easily detected change in phenotype.

One may also provide for expression of the *patched* gene or variants thereof in cells or tissues where it is not normally expressed or at abnormal times of development. Thus, mouse models of spina bifida or abnormal motor neuron differentiation in the developing spinal cord are made available. In addition, by providing expression of *ptc* protein in cells in which it is otherwise not normally produced, one can induce changes in cell behavior, e.g. through *ptc* mediated transcription modulation.

DNA constructs for homologous recombination will comprise at least a portion of the *patched or hedgehog* gene with the desired genetic modification, and will include regions of homology to the target locus. DNA constructs for random integration need not include regions of homology to mediate recombination. Conveniently, markers for positive and negative selection are included. Methods for generating cells having targeted gene modifications through homologous recombination are known in the art. For various techniques for transfecting mammalian cells, see Keown *et al.* (1990) Methods in Enzymology 185:527-537.

For embryonic stem (ES) cells, an ES cell line may be employed, or ES cells may be obtained freshly from a host, e.g. mouse, rat, guinea pig, etc. Such cells are grown on an appropriate fibroblast-feeder layer or grown in the presence of leukemia inhibiting factor (LIF). When ES cells have been transformed, they may be used to produce transgenic animals. After transformation, the cells are plated onto a feeder layer in an appropriate medium. Cells containing the construct may be detected by employing a selective medium. After sufficient time for colonies to grow, they are picked and analyzed for the occurrence of homologous recombination or integration of the construct. Those colonies that are positive may then be used for embryo manipulation and blastocyst injection. Blastocysts are obtained from 4 to 6 week old superovulated females. The ES cells are trypsinized, and the modified cells are injected into the blastocoel of the blastocyst. After injection, the blastocysts are returned to each uterine horn of pseudopregnant females. Females are then allowed to go to term and the resulting litters screened for mutant cells having the construct. By providing for a different phenotype of the blastocyst and the ES cells, chimeric progeny can be readily detected.

The chimeric animals are screened for the presence of the modified gene and males and females having the modification are mated to produce homozygous progeny. If the gene alterations cause lethality at some point in development, tissues or organs can be maintained as allogeneic or congenic grafts or transplants, or in *in vitro* culture. The transgenic animals may be any non-human mammal, such as laboratory animals, domestic animals, etc. The transgenic animals may be used in functional studies, drug screening, etc., e.g. to determine the effect of a candidate drug on basal cell carcinomas.

The subject gene may be employed for producing all or portions of the patched protein. For expression, an expression cassette may be employed, providing for a transcriptional and translational initiation region, which may be inducible or constitutive, the coding region under the transcriptional control of the transcriptional initiation region, and a transcriptional and translational termination region. Various transcriptional initiation regions may be employed which are functional in the expression host.

Specific *ptc* peptides of interest include the extracellular domains, particularly in the human mature protein, aa 120 to 437, and aa 770 to 1027. These peptides may be used as immunogens to raise antibodies that recognize the protein in an intact cell membrane. The cytoplasmic domains, as shown in Figure 2, (the amino terminus and carboxy terminus) are of interest in binding assays to detect ligands involved in signaling mediated by *ptc*.

The peptide may be expressed in prokaryotes or eukaryotes in accordance with conventional ways, depending upon the purpose for expression. For large scale production of the protein, a unicellular organism or cells of a higher organism, e.g. eukaryotes such as vertebrates, particularly mammals, may be used as the expression host, such as *E. coli, B, subthis, S. cerevisiae,* and the like. In many situations, it may be desirable to express the *patched* gene in a mammalian host, whereby the *patched* gene will be glycosylated, and transported to the cellular membrane for various studies.

With the availability of the protein in large amounts by employing an expression host, the protein may be isolated and purified in accordance with conventional ways. A lysate may be prepared of the expression host and the lysate purified using HPLC, exclusion chromatography, gel electrophoresis, affinity chromatography, or other purification technique. The purified protein will generally be at least about 80% pure, preferably at least about 90% pure, and may be up to and including 100% pure. By pure is intended free of other proteins, as well as cellular debris.

The polypeptide is used for the production of antibodies, where short fragments provide for antibodies specific for the particular polypeptide, whereas larger fragments or the entire gene allow for the production of antibodies over the surface of the polypeptide or protein. Antibodies may be raised to the normal or mutated forms of *ptc*- The extracellular domains of the protein are of interest as epitopes, particular antibodies that recognize

common changes found in abnormal, oncogenic *ptc*, which compromise the protein activity. Antibodies may be raised to isolated peptides corresponding to these domains, or to the native protein, e.g. by immunization with cells expressing *ptc*, immunization with liposomes having *ptc* inserted in the membrane, etc. Antibodies that recognize the extracellular domains of *ptc* are useful in diagnosis, typing and staging of human carcinomas.

Antibodies are prepared in accordance with conventional ways, where the expressed polypeptide or protein may be used as an immunogen, by itself or conjugated to known immunogenic carriers, e.g. KLH, pre-S HBsAg, other viral or eukaryotic proteins, or the like. Various adjuvants may be employed, with a series of injections, as appropriate, For monoclonal antibodies, after one or more booster injections, the spleen may be isolated, the splenocytes immortalized, and then screened for high affinity antibody binding. The immortalized cells, e.g. hybridomas, producing the desired antibodies may then be expanded. For further description, see Monoclonal Antibodies- A Laboratory Manual, Harlow and Lane eds., Cold Spring Harbor Laboratories, Cold Spring Harbor, New York, 1988. If desired, the mRNA encoding the heavy and light chains may be isolated and mutagenized by cloning in *E. coli,* and the heavy and light chains may be mixed to further enhance the affinity of the antibody.

The antibodies find particular use in diagnostic assays for developmental abnormalities, basal cell carcinomas and other tumors associated with mutations in *ptc*. Staging, detection and typing of tumors may utilize a quantitative immunoassay for the presence or absence of normal *ptc*. Alternatively, the presence of mutated forms of *ptc* may be determined. A reduction in normal *ptc* and/or presence of abnormal *ptc* is indicative that the tumor is *ptc*-associated.

A sample is taken from a patient suspected of having a *ptc*-associated tumor, developmental abnormality or BCNS. Samples, as used herein, include biological fluids such as blood, cerebrospinal fluid, tears, saliva, lymph, dialysis fluid and the like- organ or tissue culture derived fluids, and fluids extracted from physiological tissues. Also included in the term are derivatives and fractions of such fluids. Biopsy samples are of particular interest, e.g. skin lesions, organ tissue fragments, etc. Where metastasis is suspected, blood samples may be preferred. The number of cells in a sample will generally be at least about 10^3, usually at least 10^4 more usually at least about 10^5. The cells may be dissociated, in the case of solid tissues, or tissue sections may be analyzed. Alternatively a lysate of the cells may be prepared.

Diagnosis may be performed by a number of methods. The different methods all determine the absence or presence of normal or abnormal *ptc* in patient cells suspected of having a mutation in *ptc*. For example, detection may utilize staining of intact cells or histological sections, performed in accordance with conventional methods. The antibodies of

interest are added to the cell sample, and incubated for a period of time sufficient to allow binding to the epitope, usually at least about 10 minutes. The antibody may be labeled with radioisotopes, enzymes, fluorescers, chemiluminescers, or other labels for direct detection. Alternatively, a second stage antibody or reagent is used to amplify the signal. Such reagents are well-known in the art. For example, the primary antibody may be conjugated to biotin, with horseradish peroxidase-conjugated avidin added as a second stage reagent. Final detection uses a substrate that undergoes a color change in the presence of the peroxidase. The absence or presence of antibody binding may be determined by various methods, including flow cytometry of dissociated cells, microscopy, radiography, scintillation counting, etc.

An alternative method for diagnosis depends on the *in vitro* detection of binding between antibodies and *ptc* in a lysate. Measuring the concentration of *ptc* binding in a sample or fraction thereof may be accomplished by a variety of specific assays. A conventional sandwich type assay may be used. For example, a sandwich assay may first attach *ptc*-specific antibodies to an insoluble surface or support. The particular manner of binding is not crucial so long as it is compatible with the reagents and overall methods of the invention They may be bound to the plates covalently or non-covalently, preferably non-covalently.

The insoluble supports may be any compositions to which polypeptides can be bound, which is readily separated from soluble material, and which is otherwise compatible with the overall method. The surface of such supports may be solid or porous and of any convenient shape. Examples of suitable insoluble supports to which the receptor is bound include beads, e.g. magnetic beads, membranes and microtiter plates. These are typically made of glass, plastic (e.g. polystyrene), polysaccharides, nylon or nitrocellulose. Microtiter plates are especially convenient because a large number of assays can be carried out simultaneously, using small amounts of reagents and samples.

Patient sample lysates are then added to separately assayable supports (for example, separate wells of a microtiter plate) containing antibodies. Preferably, a series of standards, containing known concentrations of normal and/or abnormal *ptc* is assayed in parallel with the samples or aliquots thereof to serve as controls. Preferably, each sample and standard will be added to multiple wells so that mean values can be obtained for each. The incubation time should be sufficient for binding, generally, from about 0.1 to 3 hr is sufficient. After incubation, the insoluble support is generally washed of non-bound components. Generally, a dilute non-ionic detergent medium at an appropriate pH, generally 7-8, is used as a wash medium. From one to six washes may be employed, with sufficient volume to thoroughly wash nonspecifically bound proteins present in the sample.

After washing, a solution containing a second antibody is applied. The antibody will bind *ptc* with sufficient specificity such that it can be distinguished from other components present. The second antibodies may be labeled to facilitate direct, or indirect quantification of binding. Examples of labels that permit direct measurement of second receptor binding include radiolabels, such as ^{3}H or ^{125}I, fluorescers, dyes, beads, chemilumninescers, colloidal particles, and the like. Examples of labels which permit indirect measurement of binding include enzymes where the substrate may provide for a colored or fluorescent product. In a preferred embodiment, the antibodies are labeled with a covalently bound enzyme capable of providing a detectable product signal after addition of suitable substrate. Examples of suitable enzymes for use in conjugates include horseradish peroxidase, alkaline phosphatase, malate dehydrogenase and the like. Where not commercially available, such antibody-enzyme conjugates are readily produced by techniques known to those skilled in the art. The incubation time should be sufficient for the labeled ligand to bind available molecules. Generally, from about 0. 1 to 3 hr is sufficient, usually 1 hr sufficing.

After the second binding step, the insoluble support is again washed free of non-specifically bound material. The signal produced by the bound conjugate is detected by conventional means. Where an enzyme conjugate is used, an appropriate enzyme substrate is provided so a detectable product is formed.

Other immunoassays are known in the art and may find use as diagnostics. Ouchterlony plates provide a simple determination of antibody binding. Western blots may be performed on protein gels or protein spots on filters, using a detection system specific for *ptc* as desired, conveniently using a labeling method as described for the sandwich assay.

Other diagnostic assays of interest are based on the functional properties of *ptc* protein itself. Such assays are particularly useful where a large number of different sequence changes lead to a common phenotype, i.e., loss of protein function leading to oncogenesis or developmental abnormality. For example, a functional assay may be based on the transcriptional changes mediated by *hedgehog* and *patched* gene products. Addition of soluble Hh to embryonic stem cells causes induction of transcription in target genes. The presence of functional *ptc* can be determined by its ability to antagonize Hh activity. Other functional assays may detect the transport of specific molecules mediated by *ptc*, in an intact cell or membrane fragment. Conveniently, a labeled substrate is used, where the transport in or out of the cell can be quantitated by radiography, microscopy, flow cytometry, spectrophotometry, etc. Other assays may detect conformational changes, or changes in the subcellular localization of *patched* protein.

By providing for the production of large amounts of patched protein, one can identify ligands or substrates that bind to, modulate or mimic the action of *patched.* A common feature in basal cell carcinoma is the loss of adhesion between epidermal and dermal layers,

indicating a role for *ptc* in maintaining appropriate cell adhesion. Areas of investigation include the development of cancer treatments, wound healing, adverse effects of aging, metastasis, etc.

Drug screening identifies agents that provide a replacement for *ptc* function in abnormal cells. The role of *ptc* as a tumor suppressor indicates that agents which mimic its function, in terms of transmembrane transport of molecules, transcriptional down-regulation, etc., will inhibit the process of oncogenesis. These agents may also promote appropriate cell adhesion in wound healing and aging, to reverse the loss of adhesion observed in metastasis, etc. Conversely, agents that reverse *ptc* function may stimulate controlled growth and healing. Of particular interest are screening assays for agents that have a low toxicity for human cells. A wide variety of assays may be used for this purpose, including labeled *in vitro* protein-protein binding assays, electrophoretic mobility shift assays, immunoassays for protein binding, and the like. The purified protein may also be used for determination of three-dimensional crystal structure, which can be used for modeling intermolecular interactions, transporter function, etc.

The term "agent" as used herein describes any molecule, e.g. protein or pharmaceutical, with the capability of altering or mimicking the physiological function of *patched*. Generally a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a differential response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e. at zero concentration or below the level of detection.

Candidate agents encompass numerous chemical classes, though typically they are organic molecules, preferably small organic compounds having a molecular weight of more than 50 and less than about 2,500 daltons. Candidate agents comprise functional groups necessary for structural interaction with proteins, particularly hydrogen bonding, and typically include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups. The candidate agents often comprise cyclical carbon or heterocyclic structures and/or aromatic or polyaromatic structures substituted with one or more of the above functional groups. Candidate agents are also found among biomolecules including peptides, saccharides, fatty acids, steroids, purines, pyrimidines, derivatives, structural analogs or a combinations thereof.

Candidate agents are obtained from a wide variety of sources including libraries of synthetic or natural compounds. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides and oligopeptides. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural or synthetically produced libraries and compounds are

readily modified through conventional chemical, physical and biochemical means, and may be used to produce combinatorial libraries. Known pharmacological agents may be subjected to directed or random chemical modifications, such as acylation, alkylation, esterification, amidification, etc. to produce structural analogs.

Where the screening assay is a binding assay, one or more of the molecules may be joined to a label, where the label can directly or indirectly provide a detectable signal. Various labels include radioisotopes, fluorescers, chemiluminescers, enzymes, specific binding molecules, particles, e.g. magnetic particles, and the like. Specific binding molecules include pairs, such as biotin and streptavidin, digoxin and antidigoxin etc. For the specific binding members, the complementary member would normally be labeled with a molecule that provides for detection, in accordance with known procedures.

A variety of other reagents may be included in the screening assay. These include reagents like salts, neutral proteins, e.g. albumin, detergents, etc. that are used to facilitate optimal protein-protein binding and/or reduce nonspecific or background interactions. Reagents that improve the efficiency of the assay, such as protease inhibitors, nuclease inhibitors, anti-microbial agents, etc. may be used. The mixture of components are added in any order that provides for the requisite binding. Incubations are performed at any suitable temperature, typically between 4° and 40° C. Incubation periods are selected for optimum activity, but may also be optimized to facilitate rapid high-throughput screening. Typically between 0.1 and 1 hours will be sufficient.

Other assays of interest detect agents that mimic patched function, such as repression of target gene transcription, transport of patched substrate compounds, etc. For example, an expression construct comprising a *patched* gene may be introduced into a cell line under conditions that allow expression. The level of patched activity is determined by a functional assay, as previously described. In one screening assay, candidate agents are added in combination with a Hh protein, and the ability to overcome Hh antagonism of *ptc* is detected. In another assay, the ability of candidate agents to enhance *ptc* function is determined. Alternatively, candidate agents are added to a cell that lacks functional *ptc*, and screened for the ability to reproduce *ptc* in a functional assay.

In one embodiment, the drug screening assay is a cell-based assay which detects the ability of a compound to alter *patched*-dependent gene transcription. By selecting transcriptional regulatory sequences from genes whose expression is regulated by *patched* signal transduction, e.g. from *patched, GLI, hedgehog* or PTHrP genes, e.g., regulatory sequences that are responsible for the up- or down regulation of these genes in response to *patched* signalling, and operatively linking such promoters to a reporter gene, one can derive a transcription based assay which is sensitive to the ability of a specific test compound to modify *patched* signalling pathways. Expression of the reporter gene, thus, provides a

valuable screening tool for the development of compounds that act as agonists or antagonists of *patched*.

Reporter gene based assays of this invention measure the end stage of the above described cascade of events, e.g., transcriptional modulation. Accordingly, in practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on *ptc* signaling. To identify potential regulatory elements responsive to *ptc* signaling present in the transcriptional regulatory sequence of a target gene, nested deletions of genomic clones of the target gene can be constructed using standard techniques. See, for example, Current Protocols in Molecular Biology, Ausubel, F.M. et al. (eds.) Greene Publishing Associates, (1989); U.S. Patent 5,266,488; Sato et al. (1995) *J Biol Chem* 270:10314-10322; and Kube et al. (1995) *Cytokine* 7:1-7. A nested set of DNA fragments from the gene's 5'-flanking region are placed upstream of a reporter gene, such as the luciferase gene, and assayed for their ability to direct reporter gene expression in *patched* expressing cells. Host cells transiently transfected with reporter gene constructs can be scored for the induction of expression of the reporter gene in the presence and absence of *hedgehog* to determine regulatory sequences which are responsice to *patched*-dependent signalling.

In practicing one embodiment of the assay, a reporter gene construct is inserted into the reagent cell in order to generate a detection signal dependent on second messengers generated by induction with *hedgehog* protein. Typically, the reporter gene construct will include a reporter gene in operative linkage with one or more transcriptional regulatory elements responsive to the *hedgehog* activity, with the level of expression of the reporter gene providing the *hedgehog*-dependent detection signal. The amount of transcription from the reporter gene may be measured using any method known to those of skill in the art to be suitable. For example, mRNA expression from the reporter gene may be detected using RNAse protection or RNA-based PCR, or the protein product of the reporter gene may be identified by a characteristic stain or an intrinsic activity. The amount of expression from the reporter gene is then compared to the amount of expression in either the same cell in the absence of the test compound (or *hedgehog*) or it may be compared with the amount of transcription in a substantially identical cell that lacks the target receptor protein. Any statistically or otherwise significant difference in the amount of transcription indicates that the test compound has in some manner altered the signal transduction of the *patched* protein, e.g., the test compound is a potential *ptc* therapeutic.

As described in further detail below, in preferred embodiments the gene product of the reporter is detected by an intrinsic activity associated with that product. For instance, the reporter gene may encode a gene product that, by enzymatic activity, gives rise to a detection signal based on color, fluorescence, or luminescence. In other preferred embodiments, the

reporter or marker gene provides a selective growth advantage, e.g., the reporter gene may enhance cell viability, relieve a cell nutritional requirement, and/or provide resistance to a drug.

Preferred reporter genes are those that are readily detectable. The reporter gene may also be included in the construct in the form of a fusion gene with a gene that includes desired transcriptional regulatory sequences or exhibits other desirable properties. Examples of reporter genes include, but are not limited to CAT (chloramphenicol acetyl transferase) (Alton and Vapnek (1979), Nature 282: 864-869) luciferase, and other enzyme detection systems, such as beta-galactosidase; firefly luciferase (deWet et al. (1987), Mol. Cell. Biol. 7:725-737); bacterial luciferase (Engebrecht and Silverman (1984), PNAS 1: 4154-4158; Baldwin et al. (1984), Biochemistry 23: 3663-3667); alkaline phosphatase (Toh et al. (1989) Eur. J. Biochem. 182: 231-238, Hall et al. (1983) J. Mol. Appl. Gen. 2: 101), human placental secreted alkaline phosphatase (Cullen and Malim (1992) Methods in Enzymol. 216:362-368).

Transcriptional control elements which may be included in a reporter gene construct include, but are not limited to, promoters, enhancers, and repressor and activator binding sites. Suitable transcriptional regulatory elements may be derived from the transcriptional regulatory regions of genes whose expression is induced after modulation of a *patched* signal transduction pathway. The characteristics of preferred genes from which the transcriptional control elements are derived include, but are not limited to, low or undetectable expression in quiescent cells, rapid induction at the transcriptional level within minutes of extracellular simulation, induction that is transient and independent of new protein synthesis, subsequent shut-off of transcription requires new protein synthesis, and mRNAs transcribed from these genes have a short half-life. It is not necessary for all of these properties to be present.

The compounds having the desired pharmacological activity may be administered in a physiologically acceptable carrier to a host for treatment of cancer or developmental abnormalities attributable to a defect in *patched* function. The compounds may also be used to enhance *patched* function in wound healing, aging, etc. The inhibitory agents may be administered in a variety of ways, orally, topically, parenterally e.g. subcutaneously, intraperitoneally, by viral infection, intravascularly, etc. Topical treatments are of particular interest. Depending upon the manner of introduction, the compounds may be formulated in a variety of ways. The concentration of therapeutically active compound in the formulation may vary from about 0.1-100 wt.%.

The pharmaceutical compositions can be prepared in various forms, such as granules, tablets, pills, suppositories, capsules, suspensions, salves, lotions and the like. Pharmaceutical grade organic or inorganic carriers and/or diluents suitable for oral and topical use can be used to make up compositions containing the therapeutically-active compounds. Diluents known to the art include aqueous media, vegetable and animal oils and fats. Stabilizing

agents, wetting and emulsifying agents, salts for varying the osmotic pressure or buffers for securing an adequate pH value, and skin penetration enhancers can be used as auxiliary agents.

The gene or fragments thereof may be used as probes for identifying the 5' non-coding region comprising the transcriptional initiation region, particularly the enhancer regulating the transcription of *patched.* By probing a genomic library, particularly with a probe comprising the 5' coding region, one can obtain fragments comprising the 5' non-coding region. If necessary, one may walk the fragment to obtain further 5' sequence to ensure that one has at least a functional portion of the enhancer. It is found that the enhancer is proximal to the 5' coding region, a portion being in the transcribed sequence and downstream from the promoter sequences. The transcriptional initiation region may be used for many purposes, studying embryonic development, providing for regulated expression of *patched* protein or other protein of interest during embryonic development or thereafter, and in gene therapy.

The gene may also be used for gene therapy. Vectors useful for introduction of the gene include plasmids and viral vectors. Of particular interest are retroviral-based vectors, e.g. moloney murine leukemia virus and modified human immunodeficiency virus-adenovirus vectors, etc. Gene therapy may be used to treat skin lesions, an affected fetus, etc., by transfection of the normal gene into embryonic stem cells or into other fetal cells. A wide variety of viral vectors can be employed for transfection and stable integration of the gene into the genome of the cells. Alternatively, micro-injection may be employed, fusion, or the like for introduction of genes into a suitable host cell. See, for example, Dhawan *et al.* (1991) Science 254:1509-1512 and Smith *et al.* (1 990) Molecular and Cellular Biology 3268-3271.

The following examples are offered by illustration not by way of limitation.

EXPERIMENTAL

Methods and Materials

PCR on Mosquito (Anopheles gambiae) Genomic DNA. PCR primers were based on amino acid stretches of fly *ptc* that were not likely to diverge over evolutionary time and were of low degeneracy. Two such primers (P2Rl (SEO ID NO-14)-GGACGAATTCAARGTNCAYCARYTNTGG, P4Rl: (SEQ ID NO:15) GGACGAATTCCYTCCCARAARCANTC, (the underlined sequences are Eco RI linkers) amplified an appropriately sized band from mosquito genomic DNA using the PCR. The program conditions were as follows:

94 C 4 min.; 72 C Add Taq;

[49 C 30 sec.; 72 C 90 sec.; 94 C 15 sec] 3 times

[94 C 15 sec.; 50 C 30 sec.; 72 C 90 sec] 35 times

72 C 10 min; 4 C hold

This band was subcloned into the EcoRV site of pBluescript II and sequenced using the USB Sequence kit.

Screen of a Butterfly cDNA Library with Mosquito PCR Product. Using the mosquito PCR product (SEQ ID NO:7) as a probe, a 3 day embryonic *Precis coenia* gt10 cDNA library (generously provided by Sean Carroll) was screened. Filters were hybridized at 65° C overnight in a solution containing 5xSSC, 10% dextran sulfate, 5x Denhardt's, 200 µg/ml sonicated salmon sperm DNA, and 0.5% SDS. Filters were washed in 0.1X SSC, 0.1% SDS at room temperature several times to remove nonspecific hybridization. Of the 100,000 plaques initially screened, 2 overlapping clones, Ll and L2, were isolated, which corresponded to the N terminus of butterfly *ptc*. Using L2 as a probe, the library filters were rescreened and 3 additional clones (L5, L7, L8) were isolated which encompassed the remainder of the *ptc* coding sequence. The full length sequence of butterfly *ptc* (SEQ ID NO:3) was determined by ABI automated sequencing.

Screen of a Tribolium (beetle) Genomic Library with Mosquito PCR Product and 900 bp Fragment from the Butterfly Clone. A gem11 genomic library from *Tribolium casteneum* (gift of Rob Dennell) was probed with a mixture of the mosquito PCR (SEQ ID NO:7) product and BstXI/EcoRI fragment of L2. Filters were hybridized at 55 C overnight and washed as above. Of the 75,000 plaques screened, 14 clones were identified and the SacI fragment of T8 (SEQ ID NO:1), which crosshybridized with the mosquito and butterfly probes, was subcloned into pBluescript.

PCR on Mouse cDNA Using Degenerate Primers Derived from Regions Conserved in the Four Insect Homologues. Two degenerate PCR primers (P4REV- (SEQ ID NO:16) GGACGAATTCYTNGANTGYTTYTGGGA- P22- (SEQ ID NO:17) CATACCAGCCAAG CTTGTCIGGCCARTGCAT) were designed based on a comparison of *ptc* amino acid sequences from fly *(Drosophila melanogaster)* (SEQ ID NO:6), mosquito *(Anopheles gambiae)* (SEQ ID NO:8), butterfly (*Precis coenia*) (SEQ ID NO:4), and beetle *(Tribolium casteneum)* (SEQ ID NO:2). I represents inosine, which can form base pairs with all four nucleotides. P22 was used to reverse transcribe RNA from 12.5 dpc mouse limb bud (gift from David Kingsley) for 90 min at 37 C. PCR using P4REV (SEQ ID NO:17) and P22 (SEQ ID NO:18) was then performed on 1 1 of the resultant cDNA under the following conditions:

94 C 4 min.; 72 C Add Taq;

[94 C 15 sec.- 50 C 30 sec.- 72 C 90 sec.] 35 times

72 C 10 min.-, 4 C hold

PCR products of the expected size were subcloned into the TA vector (Invitrogen) and sequenced with the Sequenase Version 2.0 DNA Sequencing Kit (U. S. B.).

Using the cloned mouse PCR fragment as a probe, 300,000 plaques of a mouse 8.5 dpc gtl0 cDNA library (a gift from Brigid Hogan) were screened at 65 C as above and washed in 2x SSC, 0.1% SDS at room temperature. 7 clones were isolated, and three (M2, M4, and M8) were subcloned into pBluescript II. 200,000 plaques of this library were rescreened using first, a 1.1 kb EcoRI fragment from M2 to identify 6 clones (M9-Ml6) and secondly a mixed probe containing the most N terminal (Xhol fragment from M2) and most C terminal sequences (BamHI/BgIII fragment from M9) to isolate 5 clones (Ml7-M21). M9, M10, M14, and M17-21 were subcloned into the EcoRI site of pBluescript II (Strategene).

RNA Blots and in situ Hybridizations in Whole and Sectioned Mouse Embryos:

Northerns. A mouse embryonic Northern blot and an adult multiple tissue Northern blot (obtained from Clontech) were probed with a 900 bp EcoRl fragment from an N terminal coding region of mouse *ptc*. Hybridization was performed at 65° C in 5x SSPE, l0x Denhardt's, 100 µg/ml sonicated salmon sperm DNA, and 2% SDS. After several short room temperature washes in 2x SSC, 0.05% SDS, the blots were washed at high stringency in 0. 1 X SSC, 0.1% SDS at 50° C.

In situ hybridization of sections: 7.75, 8.5, 11.5, and 13.5 dpc mouse embryos were dissected in PBS and frozen in Tissue-Tek medium at -80° C. 12-16 µm frozen sections were cut, collected onto VectaBond (Vector Laboratories) coated slides, and dried for 30-60 minutes at room temperature. After a 10 minute fixation in 4% paraformaldehyde in PBS, the slides were washed 3 times for 3 minutes in PBS, acetylated for 10 minutes in 0.25% acetic anhydride in triethanolamine, and washed three more times for 5 minutes in PBS. Prehybridization (50% formamide, 5X SSC, 250 µg/ml yeast tRNA, 500 µg/ml sonicated salmon sperm DNA, and 5x Denhardt's) was carried out for 6 hours at room temperature in 50% formamide/5x SSC humidified chambers. The probe, which consisted of 1 kb from the N-terminus of *ptc*, was added at a concentration of 200-1000 ng/ml into the same solution used for prehybridization, and then denatured for five minutes at 80° C. Approximately 75 µl of probe were added to each slide and covered with Parafilm. The slides were incubated overnight at 65° C in the same humidified chamber used previously. The following day, the probe was washed successively in 5X SSC (5 minutes, 65° C), 0.2X SSC (1 hour, 65° C), and 0.2X SSC (10 minutes, room temperature). After five minutes in buffer Bl (0.lM maleic acid, 0.15 M NaCl, pH 7.5), the slides were blocked for 1 hour at room temperature in 1% blocking reagent (Boerhinger-Mannheim) in buffer Bl, and then incubated for 4 hours in buffer Bl containing the DIG-AP conjugated antibody (Boerhinger-Mannheim) at a 1:5000 dilution. Excess antibody was removed during two 15 minute washes in buffer Bl, followed by five

minutes in buffer B3 (100 mM Tris, l00mM NaCl, 5mM MgCl2, pH 9.5). The antibody was detected by adding an alkaline phosphatase substrate (350 µl 75 mg/ml X-phosphate in DMF, 450 µl 50 mg/ml NBT in 70% DMF in 100 mls of buffer B3) and allowing the reaction to proceed overnight in the dark. After a brief rinse in 10 mM Tris, 1mM EDTA, pH 8.0, the slides were mounted with Aquamount (Lerner Laboratories).

Drosophila 5-transcriptional initiation region -gal constructs. A series of constructs were designed that link different regions of the *ptc* promoter from *Drosophila* to a LacZ reporter gene in order to study the cis regulation of the *ptc* expression pattern. See Fig. 1. A 10.8kb BamHI/BspMl fragment comprising the 5'-non-coding region of the mRNA at its 3'-terminus was obtained and truncated by restriction enzyme digestion as shown in Fig. 1. These expression cassettes were introduced into *Drosophila* lines using a P-element vector (Thummel *et al.* (1988) Gene 74:445-456), which were injected into embryos, providing flies which could be grown to produce embryos. (See Spradling and Rubin (1982) Science 218:341-347 for a description of the procedure.) The vector used a pUC8 background into which was introduced the white gene to provide for yellow eyes, portions of the P-element for integration, and the constructs were inserted into a polylinker upstream from the LacZ gene. The resulting embryos, larvae, and adults were stained using antibodies to LacZ protein conjugated to HRP and the samples developed with OPD dye to identify the expression of the LacZ gene. The staining pattern in embryos is described in Fig. 1, indicating whether there was staining during the early and late development of the embryo.

Isolation of a Mouse ptc Gene. Homologues of fly *ptc* (SEQ ID NO:6) were isolated from three insects: mosquito, butterfly and beetle, using either PCR or low stringency library screens. PCR primers to six amino acid stretches of *ptc* of low mutatability and degeneracy were designed. One primer pair, P2 and P4, amplified an homologous fragment of *ptc* from mosquito genomic DNA that corresponded to the first hydrophilic loop of the protein. The 345bp PCR product (SEQ ID NO:7) was subcloned and sequenced and when aligned to fly *ptc*, showed 67% amino acid identity.

The cloned mosquito fragment was used to screen a butterfly gt 10 cDNA library. Of 100,000 plaques screened, five overlapping clones were isolated and used to obtain the full length coding sequence. The butterfly *ptc* homologue (SEQ ID NO:4) is 1,311 amino acids long and overall has 50% amino acid identity (72% similarity) to fly *ptc*. With the exception of a divergent C-terminus, this homology is evenly spread across the coding sequence. The mosquito PCR clone (SEQ ID NO:7) and a corresponding fragment of butterfly cDNA were used to screen a beetle gemll genomic library. Of the plaques screened, 14 clones were identified. A fragment of one clone (T8), which hybridized with the original probes, was subcloned and sequenced. This 3kb piece contains an 89 amino acid exon (SEQ ID NO:2)

which is 44% and 51% identical to the corresponding regions of fly and butterfly *ptc* respectively.

Using an alignment of the four insect homologues in the first hydrophilic loop of the *ptc*, two PCR primers were designed to a five and six amino acid stretch which were identical and of low degeneracy. These primers were used to isolate the mouse homologue using RT-PCR on embryonic limb bud RNA. An appropriately sized band was amplified and upon cloning and sequencing, it was found to encode a protein 65% identical to fly *ptc*. Using the cloned PCR product and subsequently, fragments of mouse *ptc* cDNA, a mouse embryonic cDNA library was screened. From about 300,000 plaques, 17 clones were identified and of these, 7 form overlapping cDNA's that comprise most of the protein-coding sequence (SEQ ID NO:9).

Developmental and Tissue Distribution of Mouse ptc RNA. In both the embryonic and adult Northern blots, the *ptc* probe detects a single 8kb message. Further exposure does not reveal any additional minor bands. Developmentally, *ptc* mRNA is present in low levels as early as 7 dpc and becomes quite abundant by 11 and 15 dpc. While the gene is still present at 17 dpc, the Northern blot indicates a clear decrease in the amount of message at this stage. In the adult, *ptc* RNA is present in high amounts in the brain and lung, as well as in moderate amounts in the kidney and liver. Weak signals are detected in heart, spleen, skeletal muscle, and testes.

In situ Hybridization of Mouse ptc in Whole and Section Embryos. Northern analysis indicates that *ptc* mRNA is present at 7 dpc, while there is no detectable signal in sections from 7.75 dpc embryos. This discrepancy is explained by the low level of transcription. In contrast, *ptc* is present at high levels along the neural axis of 8.5 dpc embryos. By 11.5 dpc, *ptc* can be detected in the developing lung buds and gut, consistent with its adult Northern profile. In addition, the gene is present at high levels in the ventricular zone of the central nervous system, as well as in the zona limitans of the prosencephalon. *ptc* is also strongly transcribed in the condensing cartilage of 11.5 and 13.5 dpc limb buds, as well as in the ventral portion of the somites, a region which is prospective sclerotome and eventually forms bone in the vertebral column. *ptc* is present in a wide range of tissues from endodermal, mesodermal and ectodermal origin supporting its fundamental role in embryonic development.

Isolation of the Human ptc Gene. To isolate human *ptc* (h*ptc*), 2 x 105 plaques from a human lung cDNA library (HL3022a, Clonetech) were screened with a lkbp mouse *ptc* fragment, M2-2. Filters were hybridized overnight at reduced stringency (60° C in 5X SSC, 10% dextran sulfate, 5X Denhardt's, 0.2 mg/ml sonicated salmon sperm DNA, and 0.5% SDS). Two positive plaques (Hl and H2) were isolated, the inserts cloned into pBluescript, and upon sequencing, both contained sequence highly similar to the mouse *ptc* homolog. To

isolate the 5' end, an additional 6 x 105 plaques were screened in duplicate with M2-3 EcoRI and M2-3 Xho I (containing 5' untranslated sequence of mouse *ptc*) probes. Ten plaques were purified and of these, inserts were subcloned into pBluescript. To obtain the full coding sequence, H2 was fully and H14, H20, and H21 were partially sequenced. The 5.lkbp of human *ptc* sequence (SEQ ID NO:18) contains an open reading frame of 1447 amino acids (SEQ ID NO:19) that is 96% identical and 98% similar to mouse *ptc*. The 5' and 3' untranslated sequences of human *ptc* (SEQ ID NO:18) are also highly similar to mouse *ptc* (SEQ ID NO:19) suggesting conserved regulatory sequence.

Comparison of Mouse, Human, Fly and Butterfly Sequences. The deduced mouse *ptc* protein sequence (SEQ ID NO:10) has about 38% identical amino acids to fly *ptc* over about 1,200 amino acids. This amount of conservation is dispersed through much of the protein excepting the C-terminal region. The mouse protein also has a 50 amino acid insert relative to the fly protein. Based on the sequence conservation of *ptc* and the functional conservation of *hedgehog* between fly and mouse, one concludes that *ptc* functions similarly in the two organisms. A comparison of the amino acid sequences of mouse (m*ptc*) (SEQ ID NO:10), human (h*ptc*) (SEQ ID NO:19), butterfly (b*ptc*)(SEQ ID NO:4) and *drosophila* (*ptc*) (SEQ ID NO:6) is shown in the follwing table.

ALIGNMENT OF HUMAN, MOUSE, FLY, AND BUTTERFLY *PTC* HOMOLOGS

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HPTC    MASAGNAAEPQDR--GGGGSGCIGAPGRPAGGGRRRRTGGLRRAAAPDRDYLHRPSYCDA
MPTC    MASAGNAA--------------GALGRQAGGGRRRRTGGPHRA-APDRDYLHRPSYCDA
PTC     M-----DRDSLPRVPDTHGD--VVDE---------KLFSDL---------YI-RTSWVDA
BPTC    MVAPDSEAPSNPRITAAHESPCATEA---------RHSADL---------YI-RTSWVDA
        *                                      .  ..          *. * *  **

HPTC    AFALEQISKGKATGRKAPLWLRAKFQRLLFKLGCYIQKNCGKFLVVGLLIFGAFAVGLKA
MPTC    AFALEQISKGKATGRKAPLWLRAKFQRLLFKLGCYIQKNCGKFLVVGLLIFGAFAVGLKA
PTC     QVALDQIDKGKARGSRTAIYLRSVFQSHLETLGSSVQKHAGKVLFVAILVLSTFCVGLKS
BPTC    ALALSELEKGNIEGGRTSLWIRAWLQEQLFILGCFLQGDAGKVLFVAILVLSTFCVGLKS
          ** .. **.  * .... .*. .*  *  **   * . ** * *..*....* ****.

HPTC    ANLETNVEELWVEVGGRVSRELNYTRQKIGEEAMFNPQLMIQTPKEEGANVLTTEALLQH
MPTC    ANLETNVEELWVEVGGRVSRELNYTRQKIGEEAMFNPQLMIQTPKEEGANVLTTEALLQH
PTC     AQIHSKVHQLWIQEGGRLEAELAYTQKTIGEDESATHQLLIQTTHDPNASVLHPQALLAH
BPTC    AQIHTRVDQLWVQEGGRLEAELKYTAQALGEADSSTHQLVIQTAKDPDVSLLHPGALLEH
        *.... *..**.. ***.  ** ** . .**      **.***  .   ..*   *** *

HPTC    LDSALQASRVHVYMYNRQWKLEHLCYKSGELITET-GYMDQIIEYLYPCLIITPLDCFWE
MPTC    LDSALQASRVHVYMYNRQWKLEHLCYKSGELITET-GYMDQIIEYLYPCLIITPLDCFWE
PTC     LEVLVKATAVKVHLYDTEWGLRDMCNMPSTPSFEGIYYIEQILRHLIPCSIITPLDCFWE
BPTC    LKVVHAATRVTVHMYDIEWRLKDLCYSPSIPDFEGYHHIESIIDNVIPCAIITPLDCFWE
        *     *. * * .*. .* * ..*  ..    *    .. *. . ** **********

HPTC    GAKLQSGTAYLLGKPPLR----WTNFDPLEFLEELK------KINYQVDSWEEMLNKAEV
MPTC    GAKLQSGTAYLLGKPPLR----WTNFDPLEFLEELK------KINYQVDSWEEMLNKAEV
PTC     GSQLL-GPESAVVIPGLNQRLLWTTLNPASVMQYMKQKMSEEKISFDFETVEQYMKRAAI
BPTC    GSKLL-GPDYPIYVPHLKHKLQWTHLNPLEVVEEVK-KL---KFQFPLSTIEAYMKRAGI
         *..* *     *  * *     ** ..*   .. .*      *. .   . *  ...*.
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     HPTC    GHGYMDRPCLNPADPDCPATAPNKNSTKPLDMALVLNGGCHGLSRKYMHWQEELIVGGTV
     MPTC    GHGYMDRPCLNPADPDCPATAPNKNSTKPLDVALVLNGGCQGLSRKYMHWQEELIVGGTV
     PTC     GSGYMEKPCLNPLNPNCPDTAPNKNSTQPPDVGAILSGGCYGYAAKHMHWPEELIVGGRK
   5 BPTC    TSAYMKKPCLDPTDPHCPATAPNKKSGHIPDVAAELSHGCYGFAAAYMHWPEQLIVGGAT
               .** .***.* .*.** *****.*    *..  *. ** * .   *** *.*****

     HPTC    KNSTGKLVSAHALQTMFQLMTPKQMYEHFKGYEYVSHINWNEDKAAAILEAWQRTYVEVV
     MPTC    KNATGKLVSAHALQTMFQLMTPKQMYEHFRGYDYVSHINWNEDRAAAILEAWQRTYVEVV
     PTC     RNRSGHLRKAQALQSVVQLMTEKEMYDQWQDNYKVHHLGWTQEKAAEVLNAWQRNFSREV
  10 BFTC    RNSTSALRSARALQTVVQLMGEREMYEYWADHYKVHQIGWNQEKAAAVLDAWQRKFAAEV
             .* .. *  *.***.. ***  ..**.   .   * .. * ...** .*.**** .   *

     HPTC    HQSVAQNSTQK----VLSFTTTTLDDILKSFSDVSVIRVASGYLLMLAYACLTMLRW-DC
     MPTC    HQSVAPNSTQK----VLPFTTTTLDDILKSFSDVSVIRVASGYLLMLAYACLTMLRW-DC
  15 PTC     EQLLRKQSRIATNYDIYVFSSAALDDILAKFSHPSALSIVIGVAVTVLYAFCTLLRWRDP
     BPTC    RKI-TTSGSVSSAYSFYPFSTSTLNDILGKFSEVSLKNIILGYMFMLIYVAVTLIQWRDP
              .     .          *....*.***  **. *   .  *    . *   *...* *

     HPTC    SKSQGAVGLAGVLLVALSVAAGLGLCSLIGISFNAATTQVLPFLALGVGVDDVFLLAHAF
  20 MPTC    SKSQGAVGLAGVLLVALSVAAGLGLCSLIGISFNAATTQVLPFLALGVGVDDVFLLAHAF
     PTC     VRGQSSVGVAGVLLMCFSTAAGLGLSALLGIVFNAASTQVVPFLALGLGVDHIFMLTAAY
     BPTC    IRSQAGVGIAGVLLLSITVAAGLGFCALLGIPFNASSTQIVPFLALGLGVQDMFLLTHTY

     HPTC    SETGQNKRIPFEDRTGECLKRTGASVALTSISNVTAFFMAALIPIPALRAFSLQAAVVVV
  25 MPTC    SETGQNKRIPFEDRTGECLKRTGASVALTSISNVTAFFMAALIPIPALRAFSLQAAVVVV
     PTC     AESN------RREQTKLILKKVGPSILFSACSTAGSFFAAAFIPVPALKVFCLQAAIVMC
     BPTC    VEQAGD--VPREERTGLVLKKSGLSVLLASLCNVMAFLAAALLPIPAFRVFCLQAAILLL

  30
     HPTC    FNFAMVLLIFPAILSMDLYRREDRRLDIFCCFTSPCVSRVIQVEPQAYTDTHDNTRYSPP
     MPTC    FNFAMVLLIFPAILSMDLYRPEDRRLDIFCCFTSPCVSRVIQVEPQAYTEPHSNTRYSPP
     PTC     SNLAAALLVFPAMISLDLRRRTAGRADIFCCCF-PVWKEQPKVAPPVLPLNNNNGR----
     BPTC    FNLGSILLVFPAMISLDLRRRSAAPADLLCCLM-P---ESP------LPKKKIPER----
  35

     HPTC    PPYSSHSFAHETQITMQSTVQLRTEYDPHTHVYYTTAEPRSEISVQPVTVTQDT LSCQSP
     MPTC    PPYTSHSFAHETHITMQSTVQLRTEYDPHTHVYYTTAEPRSEISVQPVTVTQDNLSCQSP
     PTC     ------ ---------------------------- GARHPKSCNNNRVPLPAQNPLLEQPA
  40 BPTC    ---------------------------------- AKTRKNDKTHRID-TTRQPLDPDVS

     HPTC    ESTSSTRDLLSQFSDSSLHCLEPPCTKWTLSSFAEKHYAPFLLKPKAKVVVIFLFLGLLG
     MPTC    ESTSSTRDLLSQFSDSSLHCLEPPCTKWTLSSFAEKHYAPFLLKPKAKVVVILLFLGLLG
  45 PTC     DIPGSS------------HSLASF----SLATFAFQHYTPFLMRSWVKFLTVMGFLAALI
     BPTC    ENVTKT------------CCL-SV----SLTKWAKNQYAPFIMRPAVKVTSMLALIAVIL

     HPTC    VSLYGTTRVRDGLDLTDIVPRETREYDFIAAQFKYFSFYNMYIVTQKA-DYPNIQHLLYD
  50 MPTC    VSLYGTTRVRDGLDLTDIVPRETREYDFIAAQFKYFSFYNMYIVTQKA-DYPNIQHLLYD
     PTC     SSLYASTRLQDGLDIIDLVPKDSNEHKFLDAQTRLFGFYSMYAVTQGNFEYPTQQQLLRD
     BPTC    TSVWGATKVKDGLDLTDIVPENTDEHEFLSRQEKYFGFYNMYAVTQGNFEYPTNQKLLYE

     HPTC    LHRSFSNVKYVMLEENKQLPKMWLHYFRDWLQGLQDAFDSDWETGKIMPNN-YKNGSDDG
  55 MPTC    LHKSFSNVKYVMLEENKQLPQMWLHYFRDWLQGLQDAFDSDWETGRIMPNN-YKNGSDDG
     PTC     YHDSFVRVPHVIKNDNGGLPDFWLLLFSEWLGNLQKIFDEEYRDGRLTKECWFPNASSDA
     BPTC    YHDQFVRIPNIIKNDNGGLTKFWLSLFRDWLLDLQVAFDKEVASGCITQEYWCKNASDEG
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Confidential

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     HPTC    VLAYKLLVQTGSRDKPIDISQLTK-QRLVDADGIINPSAFYIYLTAWVSNDPVAYAASQA
     MPTC    VLAYKLLVQTGSRDKPIDISQLTK-QRLVDADGIINPSAFYIYLTAWVSNDPVAYAASQA
     PTC     ILAYKLIVQTGHVDNPVDKELVLT-NRLVNSDGIINQRAFYNYLSAWATNDVFAYGASQG
  5  BPTC    ILAYKLMVQTGHVDNPIDKSLITAGHRLVDKDGIINPKAFYNYLSAWATNDALAYGASQG

     HPTC    NIRPHRPEWVHDKADYMPETRLRIPAAEPIEYAQFPFYLNGLRDTSDFVEAIEKVRTICS
     MPTC    NIRPHRPEWVHDKADYMPETRLRIPAAEPIEYAQFPFYLNGLRDTSDFVEAIEKVRVICN
     PTC     KLYPEPRQYFHQPNEY----DLKIPKSLPLVYAQMPFYLHGLTDTSQIKTLIGHIRDLSV
 10  BPTC    NLKPQPQRWIHSPEDV----HLEIKKSSPLIYTQLPFYLSGLSDTDSIKTLIRSVRDLCL

     HPTC    NYTSLGLSSYPNGYPFLFWEQYIGLPHWLLLFISVVLACTFLVCAVFLLNPWTAGIIVMV
     MPTC    NYTSLGLSSYPNGYPFLFWEQYISLRHWLLLSISVVLACTFLVCAVFLLNPWTAGIIVMV
 15  PTC     KYEGFGLPNYPSGIPFIFWEQYMTLRSSLAMILACVLLAALVLVSLLLLSVWAAVLVILS
     BPTC    KYEAKGLPNFPSGIPFLFWEQYLYLRTSLLLALACALGAVFIAVMVLLLNAWAAVLVTLA

     HPTC    LALMTVELFGMMGLIGIKLSAVPVVILIASVGIGVEFTVHVALAFLTAIGDKNRRAVLAL
 20  MPTC    LALMTVELFGMMGLIGIKLSAVPVVILIASVGIGVEFTVHVALAFLTAIGDKNHRAMLAL
     PTC     VLASLAQIFGAMTLLGIKLSAIPAVILILSVGMMLCFNVLISLGFMTSVGNRQRRVQLSM
     BPTC    LATLVLQLLGVMALLGVKLSAMPPVLLVLAIGRGVHFTVHLCLGFVTSIGCKRRRASLAL

     HPTC    EHMFAPVLDGAVSTLLGVLMLAGSEFDFIVRYFFAVLAILTILGVLNGLVLLPVLLSFFG
 25  MPTC    EHMFAPVLDGAVSTLLGVLMLAGSEFDFIVRYFFAVLAILTVLGVLNGLVLLPVLLSFFG
     PTC     QMSLGPLVHGMLTSGVAVFMLSTSPFEFVIPHFCWLLLVVLCVGACNSLLVFPILLSMVG
     BPTC    ESVLAPVVHGALAAALAASMLA.ASEFGFVARLFLRLLLALVFLGLIDGLLFFPIVLSILO

 30
     HPTC    PYPEVSPANGLNRLPTPSPEPPPSVVRFAMPPGHTHSGSDSSDSEYSSQTTVSGLSE-EL
     MPTC    PCPEVSPANGLNRLPTPSPEPPPSVVRFAVPPGHTNNGSDSSDSEYSSQTTVSGISE-EL
     PTC     PEAELVPLEHPDRISTPSPLPVRSSKRSGKSYVVQGSRSSRGSCQKSHHHHHKDLNDPSL
     BPTC    PAAEVRPIEHPERLSTPSPKCSPIHPRKSSSSSGGGDKSSRTS--KSAPRPC----APSL
 35

     HPTC    RHYEAQQGAGGPAHQVIVEATENPVFAHSTVVHPESRHHPPSNPRQQPHLDSGSLPPGRQ
     MPTC    RQYEAQQGAGGPAHQVIVEATENPVFARSTVVHPDSPHQPPLTPRQQPHLDSGSLSPGRQ
     PTC     TTITEEPQSWKSSNSSIQMPNDWTYQPREQ--RPASYAAPPPAYHKAAAQQHHQHQGPPT
 40  BPTC    TTITEEPSSWHSSAHSVQSSMQSIVVQPEVVVETTTYNGSDSASGRSTPTKSSHGGAITT

     HPTC    GQQPRRDPPREGLWPPLYRPRRDAFEISTEGHSGPSNRARWGPRGARSHNPPNPASTAMG
     MPTC    GQQPRRDPPREGLRPPPYRPRRDAFEISTEGHSGPSNRDRSGPRGARSHNPRNPTSTAMG
 45  PTC     TPPPPFPTA----------------YPPELQSIVVQPEVTVETTHS----------DS
     BPTC    TKVTATANIKVEVVTPSDRKSRRSYHYYDRRRDRDEDRDRDRERDRDRDRDRDRDRDRDR

     HPTC    SSVPGYCQPITTVTASASVTVAVHPPPVPGPGRNPRGGLCPGY---PETDHGLFEDPHVP
 50  MPTC    SSVPSYCQPITTVTASASVTVAVHPP--PGPGRNPRGGPCPGYESYPETDHGVFEDPHVP
     PTC     NT--------TKVTATANIKVELAMP-----GPAVRS---YNFTS--------------
     BPTC    DR--------DRERSRERDRP.DRYRD-----EPDHPA---SPRENGRDSGHE-------

 55  HPTC    FHVRCERRDSKVEVIELQDVECEERPRGSSSN
     MPTC    FHVRCERRDSKVEVIELQDVECEERPWGSSSN
     PTC     --------------------------------
     BPTC    ---------------------------- SDSSRH
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Docket No. SUV003.04

The identity of ten other clones recovered from the mouse library is not determined. These cDNAs cross-hybridize with mouse *ptc* sequence, while differing as to their restriction maps. These genes encode a family of proteins related to the patched protein. Alignment of the human and mouse nucleotide sequences, which includes coding and noncoding sequence, reveals 89% identity.

Radiation hybrid mapping of the human ptc gene. Oligonucleotide primers and conditions for specifically amplifying a portion of the human *ptc* gene from genomic DNA by the polymerase chain reaction were developed. This marker was designated STS SHGC-8725. It generates an amplification product of 196 bp, which is observed by agarose gel electrophoresis when o human DNA is used as a template, but not when rodent DNA is used. Samples were scored in duplicate for the presence or absence of the 196 bp product in 83 radiation hybrid DNA samples from the Stanford G3 Radiation Hybrid Panel (purchased from Research Genetics, Inc.) By comparison of the pattern of G3 panel scores for those with a series of Genethon meiotic linkage 5 markers, it was determined that the human *ptc* gene had a two point lod score of 1,000 with the meiotic marker D9S287, based on no radiation breaks being observed between the gene and the marker in 83 hybrid cell lines. These results indicate that the *ptc* gene lies within 50-100 kb of the marker. Subsequent physical mapping in YAC and BAC clones confirmed this close linkage estimate. Detailed map information can be obtained from http://www.shgc.stanford.edu.

Analysis of BCNS mutations. The basal cell nevus syndrome has been mapped to the same region of chromosome 9q as was found for *ptc*. An initial screen of EcoRl digested DNA from probands of 84 BCNS kindreds did not reveal major rearrangements of the *ptc* gene, and so screening was performed for more subtle sequence abnormalities. Using vectorette PCR, by the method according to Riley *et al.* (1990) N.A.R. 18:2887-2890, on a BAC that contains genomic DNA for the entire coding region of *ptc*, the intronic sequence flanking 20 of the 24 exons was determined. Single strand conformational polymorphism analysis of PCR-amplified DNA from normal individuals, BCNS o patients and sporadic basal cell carcinomas (BCC) was performed for 20 exons of *ptc* coding sequence. The amplified samples giving abnormal bands on SSCP were then sequenced.

In blood cell DNA from BCNS individuals, four independent sequence changes were found; two in exon 15 and two in exon 1 0. One 49 year old man was found to have a sequence change in exon 15. His affected sister and daughter have the same alteration, but three unafflicted relatives do not. His blood cell DNA has an insertion of 9 base pairs at nucleotide 2445 of the coding sequence, resulting in the insertion of three amino acids (PNI) after amino acid 815. Because the normal sequence preceding the insertion is also PNI, a direct repeat has been formed.

The second case of an exon 15 change is an 18 year old woman who developed jaw cysts at age 9 and BCCs at age 6. The developmental effects together with the BCCs indicate that she has BCNS, although none of her relatives are known to have the syndrome. Her blood cell DNA has a deletion of 11 bp, removing the sequence ATATCCAGCAC at nucleotides 2441 to 2452 of the coding sequence. In addition, nucleotide 2452 is changed from a T to an A. The deletion results in a frameshift that is predicted to truncate the protein after amino acid 813 with the addition of 9 amino acids. The predicted mutant protein is truncated after the seventh transmembrane domain. In *Drosophila, a ptc* protein that is truncated after the sixth transmembrane domain is inactive when ectopically expressed, in contrast to the full-length protein, suggesting that the human protein is inactivated by the exon 15 sequence change. The patient with this mutation is the first affected family member, since her parents, age 48 and 50, have neither BCCs nor other signs of the BCNS- DNA from both parents' genes have the normal nucleotide sequence for exon 15, indicating that the alteration in exon 15 arose in the same generation as did the BCNS phenotype. Hence her disease is the result of a new mutation. This sequence change is not detected in 84 control chromosomes.

Analysis of sporadic basal cell carcinomas. To determine whether *ptc* is also involved in BCCs that are not associated with the BCNS or germline changes, DNA was examined from 12 sporadic BCCS. Three alterations were found in these tumors. In one tumor, a C to T transition in exon 3 at nucleotide 523 of the coding sequence changes a highly conserved leucine to phenylalanine at residue 175 in the first putative extracellular loop domain Blood cell DNA from the same individual does not have the alteration, suggesting that it arose somatically in the tumor. SSCP was used to examine exon 3 DNA from 60 individuals who do not have BCNS, and found no changes from the normal sequence. Two other sporadic BCCs have deletions o encompassing exon 9 but not extending to exon 8.

The existence of sporadic and hereditary forms of BCCs is reminiscent of the characteristics of the two forms of retinoblastoma. This parallel, and the frequent deletion in tumors of the copy of chromosome 9q predicted by linkage to carry the wild-type allele, demonstrates that the human *ptc* is a tumor suppressor gene. *ptc* represses a variety of genes, including growth factors, during Drosophila development and may have the same effect in human skin. The often reported large body size of BCNS patients also could be due to reduced *ptc* function, perhaps due to loss of control of growth factors. The C to T transition identified in *ptc* in the sporadic BCC is also a common genetic change in the *p53* gene in BCC and is consistent with the role of sunlight in causing these tumors. By contrast, the inherited deletion and insertion mutations identified in BCNS patients, as expected, are not those characteristic of ultraviolet mutagenesis.

The identification of the *ptc* mutations as a cause of BCNS links a large body of developmental genetic information to this important human disease. In embryos lacking *ptc* function part of each body segment is transformed into an anterior-posterior mirror-image duplication of another part. The patterning changes in *ptc* mutants are due in part to derepression of another segment polarity gene, *wingless,* a homolog of the vertebrate Wnt genes that encodes secreted signaling proteins. In normal embryonic development, *ptc* repression of *wg* is relieved by the Hh signaling protein, which emanates from adjacent cells in the posterior part of each segment. The resulting localized wg expression in each segment primordium organizes the pattern of bristles on the surface of the animal. The *ptc* gene inactivates its own transcription, while Hh signaling induces *ptc* transcription.

In flies two other proteins work together with Hh to activate target genes: the ser/thr kinase *fused* and the zinc finger protein encoded by *cubitus interruptus.* Negative regulators working together with *ptc* to repress targets are *protein kinase A and costal2.* Thus, mutations that inactivate human versions of *protein kinase A or costal2,* or that cause excessive activity of human *hh, gli,* or a *fused* homolog, may modify the BCNS phenotype and be important in tumorigenesis.

In accordance with the subject invention, mammalian patched genes, including the mouse and human genes, are provided, which can serve many purposes. Mutations in the gene are found in patients with basal cell nevus syndrome, and in sporadic basal cell carcinomas. The autosomal dominant inheritance of BCNS indicates that *patched* is a tumor suppressor gene. The patched protein may be used in a screening for agonists and antagonists, and for assaying for the transcription of *ptc* mRNA. The protein or fragments thereof may be used to produce antibodies specific for the protein or specific epitopes of the protein. In addition, the gene may be employed for investigating embryonic development, by screening fetal tissue, preparing transgenic animals to serve as models, and the like.

As described above, patients with basal cell nevus syndrome have a high incidence of multiple basal cell carcinomas, medulloblastomas, and meningiomas. Because somatic *ptc* mutations have been found in sporadic basal cell carcinomas, we have screened for *ptc* mutations in several types of sporadic extracutaneous tumors. We found that 2 of 14 sporadic medulloblastomas bear somatic nonsense mutations in one copy of the gene and also deletion of the other copy. In addition, we identified mis-sense mutations in *ptc* in two of seven breast carcinomas, one of nine meningiomas, and one colon cancer cell line. No *ptc* gene mutations were detected in 10 primary colon carcinomas and eighteen bladder carcinomas.

BCNS[3] (OMIM #109400) is a rare autosomal dominant disease with diverse phenotypic abnormalities, both tumorous (BCCs, medulloblastomas, and meningiomas) and developmental (misshapen ribs, spina bifida occults, and skull abnormalities; Gorlin, R.J. (1987) Medicine 66:98-113). The BCNS gene was mapped to chromosome 9q22.3 by

linkage analysis of BCNS families and by LOH analysis in sporadic BCCs (Gallani, M.R. *et al.* (1992) Cell 69:111-117). LOH in sporadic medulloblastomas has been reported in the same chromosome region (Schofield, D. *et al.* (1995) Am J Pathol 146:472-480). Recently, the human homologue of the *Drosophila patched* (PTCII) gene has been mapped to the BCNS region (Hahn, H. *et al.* (1996) Cell 85:841-851; Johnson, R.L. *et al.* (1996) Science 272:1668-1671; Gallani, M.R. *et al.* (1996) Nat Genet 14:78-81; Xie, J. *et al.* (1997) Genes Chromosomes Cancer 18:305-309), and mutations in this gene have been found in the blood DNA of BCNS patients and in the DNA of sporadic BCCs (Hahn, H. *et al., supra;* Johnson, R.L. *et al., supra*; Gallani, M.R. *et al., supra*; and Chidambaram, A. *et al.* (1996) Cancer Res 36:4599-4601). *ptc* appears to function as a tumor suppressor gene; inactivation abrogates its normal inhibition of the hedgehog signaling pathway. Because of the wide variety of tumors in patents with the BCNS and wide tissue distribution of *ptc* gene expression, we have begun screening for *ptc* gene mutations in several types of human cancers, especially those present in increased numbers in BCNS patients (medulloblastomas), those in tissues derived embryologically from epidermis (breast carcinomas) and those with chromosome 9q LOG (bladder carcinomas; see Cairns, P. *et al.* (1993) Cancer Res 53:1230-1232; and Sidransky, D. *et al.* (1997) NEJM 326:737-740).

To further study the roles of *ptc* in development and in tumorigenesis, we have constructed mice lacking *ptc* function. By homologous recombination, part of *ptc* exon 1 (including the putative start codon) and all of exon 2 were replaced with *lacZ* and a neomycin resistance gene (Fig. 3) (DNA from the *ptc* genomic locus was isolated from a 129SV genomic phage library [Stratagene]. Exons 1-15 of human *PTC (1)* were mapped by PCR and sequencing. The 3' arm of homology was a 3.5 kb EcoRI-BamHI fragment from the second intron that gained a BamHI site from pBSII [Stratagene] and was cloned into the BamHI site of pPNT [Tybulewicz, *et al.* (1991) Cell 65:1153]. A cassette containing the gene for nuclear localized b-galactosidase, followed by the mP1 intron and polyA tail was excised from pNLacF [Mercer, *et al.* (1991) Neuron 7:703] and cloned into the Xho I site of pPNT using Xho I and Sal I linkers. The 5' arm of homology was a 6.5 kb Xho I to Nru I fragment that was cloned into the Xho I site upstream of *lacZ* via a Sal I linker. The Nru I site is in the first *ptc* exon. The resulting plasmid, KO1, was linearized with Xho I and electroporated into RI ES cells that were subjected to double selection and analyzed by Southern blot [Joyner, A.L. Gene Targeting: A Practical Approach. Oxford University Press, New York, 1993, pp.33-61]. Targeted clones were expanded and used for injection into C57Bl/6 blastocysts [Hogan, B. *et al.* Manipulating the Mouse Embryo: A Laboratory Manual Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1994, pp.196-204]. Protein made from any alternative ATG would lack the first proposed transmembrane domain, flipping the orientation of the protein in the membrane. Three independent ES clones were used to make chimeras that were bred to B6D2F1 animals to generate

heterozygous mice on a mixed background. Interbreeding of heterozygotes produced no homozygous animals among 202 offspring examined. Analysis of embryos from timed matings suggested that *ptc*$^{-/-}$ embryos die between embryonic day (E) 9.0 and E10.5, with the first gross phenotypes appearing by E8. In *ptc*$^{-/-}$ embryos, the neural tube failed to close completely and was overgrown in the head folds, hindbrain and spinal cord (Fig. 4, A to C). Embryonic lethality may have been due to abnormal development of the heart (Fig. 4B), which never beats.

In flies Ptc protein inhibits *ptc* transcription. By inhibiting Ptc function, Hh increases production of Ptc which may then bind available Hh and limit the range or duration of effective Hh signal (Y. Chen and G. Struhl, (1996) Cell 87:553). Hh signaling also post-transcriptionally regulates the zinc finger protein cubitus interruptus (ci) (C. K. Motzny and R. Holmgren, (1996) Mech Dev 52:137; Domínguez, *et al.* (1996) Science 272:1621; Hepker, *et al.* (1997) Development 124:549; Aza-Blanc, *et al.*, (1997) Cell 89:1043). In vertebrates, Sonic hedgehog (Shh) signaling induces transcription of both *ptc* and a *ci* homolog, *Gli* (Goodrich, *et al.* (1996) Genes Devel. 10:301; Marigo, *et al.* (1996) Development 122:1225; Concordet, *et al.*, (1996) Development 122:2835; Marigo, *et al.* (1996) Dev. Biol. 180:273). Derepression of *ptc* and *Gli* in *ptc*$^{-/-}$ mice should therefore reveal where Ptc is normally active.

ptc and *Gli* expression was greatly increased in *ptc*$^{-/-}$ embryos. In *ptc*$^{+/-}$ mice expression of the *lacZ* gene fused to the first *ptc* exon during targeting accurately reported the pattern of *ptc* transcription (Fig. 4, C and D). In *ptc*$^{-/-}$ embryos expression of *ptc-lacZ* was extensively derepressed starting at about E8.0 in the anterior neural tube and spreading posteriorly by E8.75 (Fig. 4, C and E). Derepression was germ layer-specific: both *ptc-lacZ* and *Gli* were expressed throughout the ectoderm and mesoderm, but not in the endoderm (Fig. 4, D to G). *ptc* expression may be excluded from the endoderm in order to avoid interfering with Shh signaling from the endoderm to the mesoderm (Roberts *et al.*, (1995) Development 121:3163). A differential requirement for Ptc may distinguish the germ layers.

As revealed by *ptc* mutants, an early site of Ptc activity is the neural tube, where Shh and Ptc act antagonistically to determine cell fates. Shh induces the floor plate and motor neurons in the ventral neural tube (Echelard *et al.*, (1993) Cell 75:1417; Roelink *et al.*, (1994) Cell 76:761; Roelink *et al.*, (1995) Cell 81:445-455). These cell types fail to form in *Shh* mutants (Chiang *et al.*, (1996) Nature 383:407). High levels of Shh produced by the notochord may induce floor plate by completely inactivating Ptc (Echelard *et al.*, (1993) *supra*; Roelink *et al.*, (1994) *supra*; Roelink *et al.*, (1995) *supra*). If so, elimination of *ptc* function might cause floor plate differentiation throughout the neural tube. Prospective floor plate cells transcribe the forkhead transcription factor *HNF3b* first and then *Shh* itself (Echelard *et al.*, (1993) *supra*; Roelink *et al.*, (1994) *supra*; Roelink *et al.*, (1995) *supra*). In

E8.5 *ptc* mutants, transcription of *HNF3b* and *Shh* was expanded dorsally (Fig. 5, A to C). Ectopic *Shh* expression was most extensive in the anterior, where transcripts could be detected throughout the neurepithelium (Fig. 5, B and C). Cells in this region were in a single layer with basal nuclei, like floor plate cells that are normally restricted to the ventral midline (Fig. 5, D and E). Expression of the intermediate neural tube marker *Pax6* (C. Walther and P. Gruss, (1991) Development 113:1435) was completely absent from *ptc* mutant embryos, suggesting that only ventral, and not ventrolateral, cell fates are specified (Fig. 5, F and G).

Dorsalizing signals from the surface ectoderm (Dickinson, *et al.* (1995) Development 121:2099; Liem, *et al.* (1995) Cell 82:969) could confer dorsal cell fates even in the absence of *ptc* function. In E8-E9 *ptc* homozygotes the dorsal neural tube marker *Pax3* was not expressed in the anterior neural tube, but was transcribed in a very small region at the dorsal-most edge of the posterior neural tube (Fig. 5, H to J). In addition *erb-b3* transcription, which marks migratory neural crest cells (Fig. 5K) (H. U. Wang and D. J. Anderson, (1997) Neuron 18:383), was not detected in the somites of *ptc* mutants (Fig. 5L). We conclude that only limited dorsal fate determination occurs in the absence of *ptc*. BMP signals maintain dorsal gene expression (Dickinson, *et al.* (1995) *supra*; Liem, *et al.* (1995) *supra*), so either *ptc* is required for BMPs to work or BMP signaling is ineffective in most cells expressing Shh targets.

Ventralization of the neural tube in *ptc* mutants occurred without affecting cell identity along the rostrocaudal axis. In $ptc^{-/-}$ embryos, cells in the anterior neural tube expressed the forebrain marker *Nkx2.1* (Shimamura, *et al.* (1995) Development 121:3923) and cells in the spinal cord transcribed low levels of *hoxb1* (Wilkinson, *et al.* (1989) Nature 341:405) (Fig. 5, M and N). *hoxb1* was not transcribed in the fourth rhombomere of *ptc* mutants (Fig. 5, N). This may reflect a transformation of hindbrain cells to floor plate, since *hoxb1* is excluded from the midline of wild-type embryos. Conversely, in the anterior, *Nkx2.1* expression was expanded dorsally in mutants compared to wild-type embryos (Fig. 5, M).

$ptc^{+/-}$ mice had phenotypes similar to those of BCNS patients: they were larger than their wild-type littermates [30.72 $\pm$ 3.83 (average $\pm$ SD; n=29) vs. 26.54 $\pm$ 2.51 (n=39) at 2-3 months; P=0.000001], a small fraction (3 of 389 mice examined) had hindlimb defects such as extra digits or syndactyly (Fig. 6A) or obvious soft tissue tumors (1 of 243) and many developed brain tumors (see below).

Of 243 $ptc^{+/-}$ mice which were between the ages of 2 and 9 months and were not sacrificed for other studies, 18 died or were euthanized because of sickness. No wild-type littermates died. Ten of the affected heterozygotes were autopsied and eight were found to have large growths in the cerebellum that resembled medulloblastomas (Fig. 6, B and C).

Human medulloblastomas are believed to arise from a "primitive neurectodermal" cell type (J.P. Provias and L. E. Becker, (1996) J Neurooncol 29:35). They are most common in children, can be metastatic or non-metastatic, and can have glial and neuronal properties. The histology of tumors from *ptc*$^{+/-}$mice was similar to that of human medulloblastoma: tumor cells were small, with dark carrot-shaped nuclei and little cytoplasm (Fig. 6, D and E), and although a subset expressed neurofilament protein and synaptophysin (Fig. 6F) (For immunostaining, two tumors were fixed and embedded in paraffin. Tissue sections (4 mm) were cleared and dehydrated, treated with 3% hydrogen peroxide and then with a dilution of 1:10 normal rabbit serum (Vector Laboratories). Anti-synaptophysin (Boehringer-Mannheim) was used at a dilution of 1:5 and anti-neurofilament protein (Dako) at 1:50. Antibody binding was visualized with a peroxidase Vectastain Elite ABC kit (Vector Laboratories). Nuclei were counterstained with hematoxylin. Like anti-synaptophysin, anti-neurofilament staining appeared in processes of the tumor cells.), the majority of cells appeared undifferentiated. Of the two autopsied animals without apparent medulloblastomas, one had a large tumor growing out of its rib muscle and the other died for unknown reasons. Medulloblastomas and soft tissue tumors were also observed in *ptc*$^{+/-}$ mice maintained on an inbred 129SV background: 6 of 27 had obvious medulloblastomas; 2 of 27 had tumors in the muscle of their leg; and 3 of 27 died but were not examined.

The *ptc* and *Gli* genes were strongly transcribed in the brain tumors but not in surrounding tissue (Fig. 7, A and B; n = 3 of 3 tumors examined). There was no detectable increase in *Shh* expression (Fig. 7C). To assess the incidence of medulloblastomas, brains from 47 asymptomatic *ptc*$^{+/-}$ mice were randomly collected and stained with X-gal. Nine brains contained medulloblastomas that were easily recognized by their disorganized morphology and intense *ptc-lacZ* expression (Fig. 7D). Medulloblastomas were observed in 7 of 23 (30.4%) *ptc*$^{+/-}$ mice at 12 to 25 weeks of age, 1 of 12 (8.3%) mice at 9 to 10 weeks and 1 of 12 (8.3%) mice at 5 weeks. Tumors can therefore arise as early as 5 weeks postnatally, but they increase in severity and frequency as the animal ages.

We looked for changes in *ptc-lacZ* expression that might reflect early stages of tumorigenesis. At all stages examined, about half of the animals [50% at 5 to 10 weeks (n=24), 56.5% at 12 to 25 weeks (n=23)] exhibited regions of increased X-gal staining on the surface of the cerebellum (Fig. 7E). These regions were usually lateral and often extended down into the fissures separating the folia (Fig. 7, E and F). The mouse medulloblastomas may arise from these cells, which are superficial to the molecular layer of the cerebellum (Fig. 7F). During fetal development, prospective cerebellar granule cells proliferate in the external granule layer (EGL), the outermost layer of the cerebellum. Granule cells then leave and migrate past the Purkinje cells to form the internal granule cell layer of the adult animal, gradually depleting the EGL. The remnants of the fetal EGL have been proposed to be a source of human medulloblastoma progenitors, a hypothesis consistent with the higher

frequency of these tumors in children (L. Stevenson and F. Echlin, (1934) Arch. Neurol. Psychiat. 31:93; Kadin, *et al.* (1970) J Neuropathol Exp Neurol 29:583).

The abundance of cerebellar *ptc* transcripts was reduced by about 50% in the $ptc^{+/-}$ mice compared to wild-type littermates (Fig. 7G). This reduction could lead to ectopic expression of Shh target genes and to uncontrolled cell proliferation. Brain tumors might arise from Ptc haploinsufficiency alone, from additional mutations in the second *ptc* allele, or from a combination of *ptc* mutations with mutations in other tumor suppressor loci. We have not observed BCCs in $ptc^{+/-}$ mice, perhaps because somatic inactivation of the second *ptc* gene is required as it is in human BCCs.

Our analysis has revealed that Ptc controls growth and pattern formation in early neural development and in the adult cerebellum. Autoregulation of *ptc* occurs in vertebrates as it does in flies, and the balance between Hh and Ptc activities appears critical for normal development. The importance of Ptc dosage is emphasized by the phenotype of the $ptc^{+/-}$ mice, which develop a tumor type observed in the corresponding human cancer predisposition syndrome. Medulloblastoma is a common childhood brain tumor and the prognosis remains grim. The Hh/Ptc pathway may provide new diagnostic tools and new insights into tumorigenesis that may be directed toward potential therapies.

Materials and methods

Clinical Materials. Diagnoses of all tumors were confirmed histologically. Cell lines were obtained from the America Type Culture Collection. DNA was extracted from tumors or matched normal tissue (peripheral blood leukocytes or skin) as described (Cogen, P.H. *et al.* (1990) Genomics 8:279-285; and Sambrook, J. *et al.* Molecular Cloning: A Laboratory Manual, Ed. 2, Vol. 2, pp. 9.17 - 9.19, Cold Spring Harbor, NY (1989)).

PCR and Heteroduplex Analysis. PCR amplification and heteroduplex/SSCP analysis were performed as described (Johnson, R.L. *et al., supra*; Spritz, R.A. *et al.* (1992) Am J Hum Genet 51:1058-1065). Primers used and intron/exon boundary sequences of the *ptc* gene were derived as reported previously (Johnson, R.L. *et al., supra*) and are shown in Table 1. Primers for exon 1 and 2 were from Hahn *et al.* (*supra*).

Sequence Analysis. Exon segments exhibiting bands were reamplified and were sequenced directly using the Sequenase sequencing kit according to the protocol recommended by the manufacturer (United States Biochemical Corp.). A second sequencing was performed using independently amplified PCR products to confirm the sequence change. The amplified PCR products from each tumor were also cloned into the plasmid vector pCR 2.1 (InVitrogen), followed by sequence analysis of at least four independent clones. The sequence alteration was confirmed from at least two independent clones. Simplified

amplification of specific allele analysis was performed according to Lei and Hall (Lei, X. and Hall, B.G. (1994) Biotechniques 16:44-45).

Allele Loss Analysis. Microsatellites used for allelic loss analysis were D9S109, DpS119, D9S127, D9S196, and D9S287 described in the CHLC human screening set (Research Genetics). A part of the *ptc* intron 1 sequence was tested for polymorphism in a control population and found to be polymorphic in 80% of the samples tested. This microsatellite was used for analysis of *ptc* gene allelic loss in bladder carcinomas. The primer sequences are as follows: forward primer, 5'-CTGAGCAGATTTCCCAGGTC-3'; and reverse primer, 5'-CCTCAGACAGACCTTTCCTC-3'. The PCR cycling for this newly isolated marker was 4 min. at 95 C, followed by 30 cycles of 40 s at 95 C, 2 min. at 60 C, and 1 min. at 72 C. PCR products were separated on 6% polyacrylamide gels and exposed to film.

Results and Discussion

Intronic boundaries were determined for 22 exons of *ptc* by sequencing vectorette PCR products derived from BAC 192J22 (Johnson R.L., *supra*; Table 1). Our findings are in agreement with those of Hahn *et al.* (*supra*), expect that we find exon 12 is composed of 2 separate exons of 126 and 119 nucleotides. This indicates that *ptc* is composed of 23 coding exons instead of 22. In addition, we find that exons 3, 4, 10, 11, 17, 21, and 23 differ slightly in size than reported previously (Hahn *et al., supra*). Of 63 tumors studied, 14 were sporadic medulloblastomas, and 9 were sporadic meningiomas. These 23 tumors were examined for allelic deletions by genotyping of tumor and blood DNA with microsatellite markers that flank the *ptc* gene: D9S119, D9S196, D9S287, D9S127, and D9S109. Four of 14 medulloblastomas had LOH. Two of the medulloblastomas, both of which had LOH, had mutations (med34 and med36; see Cogen, P.H. *et al., supra*), which are predicted to result in truncated proteins (Table 2). DNA samples from the blood of these patients lack these mutations, indicating that they both are somatic mutations. med34 also has allelic loss on 17p (Cogen, P.H. *et al., supra*). We were unable to detect *ptc* gene mutations by heteroduplex analysis in the other two medulloblastomas bearing LOH on 9q. The pathological features of these two tumors differed in that med34 belongs to the desmoplastic subtype, whereas med36 is of the classic type, indicating that *ptc* mutations in medulloblastomas are not restricted to a specific subtype.

TABLE 1 *Primers and boundary sequences of PTCH*

	5' Boundary[a]	Nucleotide position[b]	Exon size	3' boundary[a]	Reading frame[c]	Primers
1	ND[d]	ND	ND	aagGTGNAT	ND	
2	ND	202	193	aagGTAAGA	3	
3	TGTCAGg	395	190	cagGTAAGG	1	3F GAGTTTGCAGTGATGTTGCTNTTC 3R ACCGCCTTACCTGCTCCTC
4	TATTAGg	585	70	cagGTATAT	2	4F TGCACTAATTTTCTTATTACACTC 4R TAAGGCACACTACTGGGGTG
5	TGACAGg	655	92	ccaGTAAGT	3	5F GAACACCCCAGTAGTGTGCC 5R TGAGTCCTAGAGAAGTCACAGACA
6	TTGCACg	747	199	aaaGTGAGT	2	6F GGCTCTTTTCATGGTCTCGTC 6R TGTTTTGCTCTCCACCGTTC
7	TTTTAGg	945	122	cagGTAAGC	3	7F GCACTGGATTTTAACAAGGCATG 7R AGGGCATAGATTGTCCTCGG
8	CTGCAGg	1068	148	gagGTAAAC	2	8F TGGGAATACTGATGATGTGCC 8R CATAACCAGCGAGTCTGCAC
9	CCACAGg	1216	132	atgGTAACG	3	9F CATTTGGGCATTTCGCATTC 9R ACCAAACCAAACTCCAGCCC
10	TTCCAGg	1348	156	cagGTACTA	3	10F TGCCCCCATTGTTCTGCTTG 10R GGACAGCAGATAAATGGCTCC
11	CTGTAGg	1504	99	gagGTAATG	3	11F GCATCTCGCATGTCTAATGCCAC 11R AAGCTGTGATGTCCCCAAAG
12	TCCCAGg	1603	126	cagGTGAGC	3	12F GACCATGTCCAGTGCAGCTC 12R CGTTCAGGATCACCACAGCC
13	TCCCAGg	1729	119	aagGTACAT	3	13F AGTCCTCTGATTGGGGGGAG 13R CCATTCTGCACCCAATCAAAAC
14	TTTCAGg	1848	403	aagGTAATC	2	14F AAAATGGCAGAATGAAAGCACC 14R CTGATGAACTCCAAAGCTTCTG
15	TTCCAGg	2251	310	aggGTAAGA	3	15F GGAAGAGTCAGTGGTGCTCC 15R CGCCAAAGACCGAAAGCAC
16	TTCTAGg	2561	143	cagGTACTC	1	16F AGGGTCCTTCTGGCTGCGAG 16R GCTGTCAAGCAGCCTCCAC
17	TTGTAGg	2704	184	gaaGTAAGT	3	17F GCTCTCAAGGCAGAAGTCTG 17R GGAAGGCACCTCTGTAAGTAC
18	GTCCAGg	2888	281	atgGTGAGT	1	18F GCTCCTAACCTGTGCCCTTC 18R GAATTTGACTTCCACAAAGCCC
19	CTCCAGg	3169	138	ttgGTATGG	3	19F CGCCCACTGACCACTGTGTG 19R GAGCCAGAGGAAATGGGTTG
20	GCACAGg	3307	143	cagGTAAGC	3	20F AGCATTTACCAGGTGAAGTCC 20R TTGCACACGCCTGCTTAT
21	TCCCAGg	3450	100	gagGTCAGT	2	21F TGTTCCCGTTTCCTCTTG 21R GCACAGGAAACACACCATTC
22	AAATAGg	3550	255	actGTAAGT	3	22F GCAGGTAAATGGACAAGAAGCAC 22R ACTACCACGGTGGGAAGACC
23	CTGCAGg	3805	541	gagGTGAGT	3	23F CCCTTCTAACCCACCCTCAC 23R GACACATCAGCCTTGCTC
24	ND	4346	ND	ND		

[a] Consensus sequences for the 5' and 3' exonic boundaries are $(T_C)_{11}NCAGg$ and $agGT^G_AAGT$, respectively (20). Lower case denotes exonic sequence.
[b] Nucleotide positions are in reference to the coding sequence of *PTCH* (3) with the beginning ATG as nucleotide 1.
[c] Reading frame 5' exon boundary begins after the first, second, or third base of the codon of the translational reading frame.
[d] ND not determined.

One report (Schofield, D. *et al., supra*) has shown that five medulloblastomas (two BCNS-associated cases and three sporadic cases) bearing LOH on chromosome 9q22.3-q31 are all of the desmoplastic subtype, suggesting LOH on 9q22.3 is histological subtype specific. We feel that the conclusion derived from only five positive tumors is a not strong one because we and others (Raffel, C. *et al.* (1997) Cancer Res 57:842-845) have found nondesmoplastic subtypes of medulloblastomas bearing LOH on chromosome 9q22.3. Independently, another group has reported their finding of *ptc* mutations in sporadic medulloblastomas (Raffel, C. *et al, supra*).

A change of T to C at nucleotide 2990 (in exon 18) was identified in DNA from one of nine sporadic meningiomas, causing a predicted change of codon 997 from Ile to Thr (Table 2). The meningioma bearing this mutation also has allelic loss on 9q22.3. Blood cell DNA is heterozygous for this mutation, but DNA from the tumor contains only the mutant sequence. Of 100 normal chromosomes examined, none has this sequence change, suggesting that this mutation is not likely a common polymorphism. This patient is 84 years

old and has had no phenotypic abnormalities suggestive of the BCNS, suggesting that this sequence alteration may not have caused complete inactivation of the *ptc* gene. None of the other eight meningiomas had detectable LOH at chromosome 9q.

TABLE 2 *PATCHED gene alterations*[a]

Tumor	Pathology	Nucleotide	Codon	Exon	Consequence	LOH	Mutation Type
Med34	Medulloblastoma (desmoplastic)	TC1869A	623	14	Frameshift	Yes	Somatic
Med36	Medulloblastoma (classic)	G2503T	835	15	Glu to STOP	Yes	Somatic
Men1	Meningioma	T2990C	997	18	Ile to Thr	Yes	Germ-line
Br349	Breast carcinoma	T2863C	955	17	Tyr to His	Yes	Somatic
Br321	Breast carcinoma	A2975G	995	18	Glu to Gly	No	Somatic
Co320	Colon tumor cell line	A2000C	667	14	Glu to Ala	No	Unknown
Co8-1	Colon carcinoma	T to C	Intron 10		Polymorphism	No	Germ-line
Co15-1	Colon carcinoma	T to C	Intron 10		Polymorphism	No	Germ-line

We also examined a variety of other tumors (10 primary tumors and 1 cell line), 18 bladder tumors (14 primary tumors and 4 cell lines), and 2 ovarian cancer cell lines. These tumors are not known to occur in higher than expected frequency in BCNS patients. We identified sequence abnormalities in two breast carcinomas and in the one colon cancer cell line (Table 2). The mutation found in breast carcinoma Br349 is not present in the patient's normal skin DNA, indicating that the sequence change is a somatic mutation. Direct sequencing of the PCR product indicated that only the mutant allele is present in the tumor. This mutation changes codon 955 from Tyr to His, and this Tyr is conserved in human, murine, chicken, and fly *ptc*II homologues (Goodrich, L.V. *et al.* (1996) Genes Dev 10:301-312). The mutation in breast carcinoma Br321 is predicted to change codon 995 from Glu to Gly, and the tumor with this mutation retains the wild-type allele. We have sequenced exon 18 in DNA from the blood of 50 normal person s and found no changes from the published sequence, suggesting that the sequence change found in Br321 is not a common polymorphism. Furthermore, examination of the DNA from the cultured skin fibroblasts of the patient did not reveal the same mutation, indicating that this is a somatic mutation.

Because DNA is not available from normal cells of the patient from which colon cell line 320 was established, we used simplified amplification of specific allele analysis (Lei, X.

and Hall, B.G., *supra*) to examine 50 normal blood DNA samples for the presence of the sequence alteration and found none but the DNA from this cell line to have the mutant allele, suggesting that this mutation also is unlikely to be a common sequence polymorphism. For bladder carcinomas, a newly isolated microsatellite that was derived from intron 1 of the *ptc* gene was used to examine LOH in the tumor. Three primary bladder carcinomas showed LOH at this intragenic locus. With no *ptc* mutations detected in these tumors, we suspect that the LOH in these three bladder carcinomas may reflect the high incidence of while chromosome 9 loss in bladder cancers (Sidransky, D. *et al., supra*). A similar observation has been reported previously (Simoneau, A. R. *et al.* (1996) Cancer Res 56:5039-5043).

We also detected a sequence change in intron 10 in two colon carcinomas, 15-1 and 8-1, an alteration that was reported previously as a splicing mutation (Unden, A.B. *et al.* (1996) Cancer Res 56:4562-4565). Because we found the same sequence change in about 20% of normal control samples, we suggest that this more likely is a nonpathogenic polymorphism. The *ptc* protein is predicted to contain 12 transmembrane domains, two large extracellular loops, and one intracellular loop (Goodrich, L.V. *et al., supra*). Of the six mutations we identified, four are missense mutations. Three mutations lead to amino acid substitutions in the second extracellular loop, and one mutation results in an amino acid change in the intracellular domain.

Our data indicate that somatic inactivation of the *ptc* gene does occur in some sporadic medulloblastomas. In addition, because missense mutations of the *ptc* gene were detected in breast carcinomas, we suspect that defects of the *ptc* function also may be involved in some breast carcinomas, although biochemical evidence is necessary to show how these missense mutations might impair *ptc* function. Of 11 colon cancers and 18 bladder carcinomas examined, we found only one mutation in 1 colon cell line, suggesting that *ptc* gene mutations are relatively uncommon in clon and bladder cancers, although the incidence of chromosome 9 loss in bladder cancers is high (Cairns, P. *et al., supra*).

Published reports of SSCP analysis of tumor DNA identified mutations in the *ptc* gene in only 30% of sporadic BCCs, although chromosome 9q22.3 LOH was reported in more than 50% of these tumors (Gallani, M.R. *et al., supra*). It has been reported that heteroduplex/SSCP analysis of gene mutations is more sensitive than SSCP analysis (Spritz, R.A. *et al., supra*). In our studies, we were able to identify a point mutation in the 310-bp - PCR product from exon 15 using heteroduplex analysis, whereas SSCP analysis failed to reveal this sequence change (Table 2). Therefore, we suspect that there may be more mutations in BCCs than we have found thus far. Analysis of the *ptc* gene in BCNS patients and in sporadic BCCs has identified mutations scattered widely across the gene, and the majority of mutations were predicted to result in truncated proteins (Hahn, H. *et al., supra*; Johnson, R.L. *et al., supra*; Gallani, M.R. *et al., supra*; Chidambaram, A. *et al., supra*;

Unden, A.B. *et al., supra*; Wicking, C. *et al.* (1997) Am J Hum Genet 60:21-26). In our screening, we found two breast carcinomas bearing missense mutations of the *ptc* gene. In one of these two tumors, B349, direct sequencing indicated a deletion of the other copy of the *ptc* gene. Any comparison of mutations in skin cancers versus extracutaneous tumors must consider the wholly different causes of these mutations; UV light is unique to the skin.

All publications and patent applications cited in this specification are herein incorporated by reference as if each individual publication or patent o application were specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be readily apparent to those of ordinary skill in the art in light of the teachings of this invention that certain changes and modifications may be made thereto without departing from the spirit or scope of the appended claims.

SUBSTITUTE SHEET (RULE 26)

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:

(ii) TITLE OF INVENTION:

(iii) NUMBER OF SEQUENCES: 19

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Foley, Hoag & Eliot LLP
(B) STREET: One Post Office Square
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: US
(F) ZIP: 02109

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Vincent, Matthew P.
(B) REGISTRATION NUMBER: 36,709
(C) REFERENCE/DOCKET NUMBER: SUV003.04

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: 617-832-1000
(B) TELEFAX: 617-832-7000

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 736 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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TTTTTNAACC CCCCCCACCC GGAATTCCNA NTNNCCNCCC CCAAATTACA ACTCCAGNCC 180

AAAATTNANA NAATTGGTCC TAACCTAACC NATNGTTGTT ACGGTTTCCC CCCCCAAATA 240

CATGCACTGG CCCGAACACT TGATCGTTGC CGTTCCAATA AGAATAAATC TGGTCATATT 300

AAACAAGCCN AAAGCTTTAC AAACTGTTGT ACAATTAATG GGCGAACACG AACTGTTCGA 360

ATTCTGGTCT GGACATTACA AAGTGCACCA CATCGGATGG AACCAGGAGA AGGCCACAAC 420

CGTACTGAAC GCCTGGCAGA AGAAGTTCGC ACAGGTTGGT GGTTGGCGCA AGGAGTAGAG 480

TGAATGGTGG TAATTTTTGG TTGTTCCAGG AGGTGGATCG TCTGACGAAG AGCAAGAAGT 540

CGTCGAATTA CATCTTCGTG ACGTTCTCCA CCGCCAATTT GAACAAGATG TTGAAGGAGG 600

CGTCGAANAC GGACGTGGTG AAGCTGGGGG TGGTGCTGGG GGTGGCGGCG GTGTACGGGT 660

GGGTGGCCCA GTCGGGGCTG GCTGCCTTGG GAGTGCTGGT CTTNGCGNGC TNCNATTCGC 720

CCTATAGTNA GNCGTA 736

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 107 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Leu Thr Pro Xaa Val Val Thr Val Ser Pro Pro Lys Tyr Met His Trp
20 25 30

Pro Glu His Leu Ile Val Ala Val Pro Ile Arg Ile Asn Leu Val Ile
35 40 45

Leu Asn Lys Pro Lys Ala Leu Gln Thr Val Val Gln Leu Met Gly Glu
50 55 60

His Glu Leu Phe Glu Phe Trp Ser Gly His Tyr Lys Val His His Ile
65 70 75 80

Gly Trp Asn Gln Glu Lys Ala Thr Thr Val Leu Asn Ala Trp Gln Lys
85 90 95

Lys Phe Ala Gln Val Gly Gly Trp Arg Lys Glu
100 105

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5187 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GCCGCGCCGG ACCGGGACTA TCTGCACCGG CCCAGCTACT GCGACGCCGC CTTCGCTCTG 240

GAGCAGATTT CCAAGGGGAA GGCTACTGGC CGGAAAGCGC CGCTGTGGCT GAGAGCGAAG 300

TTTCAGAGAC TCTTATTTAA ACTGGGTTGT TACATTCAAA AGAACTGCGG CAAGTTTTTG 360

GTTGTGGGTC TCCTCATATT TGGGGCCTTC GCTGTGGGAT TAAAGGCAGC TAATCTCGAG 420

ACCAACGTGG AGGAGCTGTG GGTGGAAGTT GGTGGACGAG TGAGTCGAGA ATTAAATTAT 480

ACCCGTCAGA AGATAGGAGA AGAGGCTATG TTTAATCCTC AACTCATGAT ACAGACTCCA 540

AAAGAAGAAG GCGCTAATGT TCTGACCACA GAGGCTCTCC TGCAACACCT GGACTCAGCA 600

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TGCTACAAAT CAGGGGAACT TATCACGGAG ACAGGTTACA TGGATCAGAT AATAGAATAC 720

CTTTACCCTT GCTTAATCAT TACACCTTTG GACTGCTTCT GGGAAGGGGC AAAGCTACAG 780

TCCGGGACAG CATACCTCCT AGGTAAGCCT CCTTTACGGT GGACAAACTT TGACCCCTTG 840

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Docket No. 2000-0150

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(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1311 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

Document ID: US 6,080,724 A

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

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            20                  25                  30

Tyr Ile Arg Thr Ser Trp Val Asp Ala Ala Leu Ala Leu Ser Glu Leu
        35                  40                  45

Glu Lys Gly Asn Ile Glu Gly Gly Arg Thr Ser Leu Trp Ile Arg Ala
    50                  55                  60

Trp Leu Gln Glu Gln Leu Phe Ile Leu Gly Cys Phe Leu Gln Gly Asp
65                  70                  75                  80

Ala Gly Lys Val Leu Phe Val Ala Ile Leu Val Leu Ser Thr Phe Cys
                85                  90                  95

Val Gly Leu Lys Ser Ala Gln Ile His Thr Arg Val Asp Gln Leu Trp
            100                 105                 110

Val Gln Glu Gly Gly Arg Leu Glu Ala Glu Leu Lys Tyr Thr Ala Gln
        115                 120                 125

Ala Leu Gly Glu Ala Asp Ser Ser Thr His Gln Leu Val Ile Gln Thr
    130                 135                 140

Ala Lys Asp Pro Asp Val Ser Leu Leu His Pro Gly Ala Leu Leu Glu
145                 150                 155                 160

His Leu Lys Val Val His Ala Ala Thr Arg Val Thr Val His Met Tyr
                165                 170                 175

Asp Ile Glu Trp Arg Leu Lys Asp Leu Cys Tyr Ser Pro Ser Ile Pro
            180                 185                 190

Asp Phe Glu Gly Tyr His His Ile Glu Ser Ile Ile Asp Asn Val Ile
        195                 200                 205

Pro Cys Ala Ile Ile Thr Pro Leu Asp Cys Phe Trp Glu Gly Ser Lys
    210                 215                 220

Leu Leu Gly Pro Asp Tyr Pro Ile Tyr Val Pro His Leu Lys His Lys
225                 230                 235                 240

Leu Gln Trp Thr His Leu Asn Pro Leu Glu Val Val Glu Glu Val Lys
                245                 250                 255

Lys Leu Lys Phe Gln Phe Pro Leu Ser Thr Ile Glu Ala Tyr Met Lys
            260                 265                 270

Arg Ala Gly Ile Thr Ser Ala Tyr Met Lys Lys Pro Cys Leu Asp Pro
        275                 280                 285

Thr Asp Pro His Cys Pro Ala Thr Ala Pro Asn Lys Lys Ser Gly His
    290                 295                 300
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SUBSTITUTE SHEET (RULE 26)

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485 490 495

Pro Phe Leu Ala Leu Gly Leu Gly Val Gln Asp Met Phe Leu Leu Thr
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530 535 540

Cys Asn Val Met Ala Phe Leu Ala Ala Ala Leu Leu Pro Ile Pro Ala
545 550 555 560

Phe Arg Val Phe Cys Leu Gln Ala Ala Ile Leu Leu Leu Phe Asn Leu
565 570 575

Gly Ser Ile Leu Leu Val Phe Pro Ala Met Ile Ser Leu Asp Leu Arg
580 585 590

Arg Arg Ser Ala Ala Arg Ala Asp Leu Leu Cys Cys Leu Met Pro Glu
595 600 605

Ser Pro Leu Pro Lys Lys Lys Ile Pro Glu Arg Ala Lys Thr Arg Lys
610 615 620

Asn Asp Lys Thr His Arg Ile Asp Thr Thr Arg Gln Pro Leu Asp Pro
625 630 635 640

09/445,150 PCT/US98/12597

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Asp Val Ser Glu Asn Val Thr Lys Thr Cys Cys Leu Ser Val Ser Leu
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        675                 680                 685

Val Trp Gly Ala Thr Lys Val Lys Asp Gly Leu Asp Leu Thr Asp Ile
    690                 695                 700

Val Pro Glu Asn Thr Asp Glu His Glu Phe Leu Ser Arg Gln Glu Lys
705                 710                 715                 720

Tyr Phe Gly Phe Tyr Asn Met Tyr Ala Val Thr Gln Gly Asn Phe Glu
                725                 730                 735

Tyr Pro Thr Asn Gln Lys Leu Leu Tyr Glu Tyr His Asp Gln Phe Val
            740                 745                 750

Arg Ile Pro Asn Ile Ile Lys Asn Asp Asn Gly Gly Leu Thr Lys Phe
        755                 760                 765

Trp Leu Ser Leu Phe Arg Asp Trp Leu Leu Asp Leu Gln Val Ala Phe
    770                 775                 780

Asp Lys Glu Val Ala Ser Gly Cys Ile Thr Gln Glu Tyr Trp Cys Lys
785                 790                 795                 800

Asn Ala Ser Asp Glu Gly Ile Leu Ala Tyr Lys Leu Met Val Gln Thr
                805                 810                 815

Gly His Val Asp Asn Pro Ile Asp Lys Ser Leu Ile Thr Ala Gly His
            820                 825                 830

Arg Leu Val Asp Lys Asp Gly Ile Ile Asn Pro Lys Ala Phe Tyr Asn
        835                 840                 845

Tyr Leu Ser Ala Trp Ala Thr Asn Asp Ala Leu Ala Tyr Gly Ala Ser
    850                 855                 860

Gln Gly Asn Leu Lys Pro Gln Pro Gln Arg Trp Ile His Ser Pro Glu
865                 870                 875                 880

Asp Val His Leu Glu Ile Lys Lys Ser Ser Pro Leu Ile Tyr Thr Gln
                885                 890                 895

Leu Pro Phe Tyr Leu Ser Gly Leu Ser Asp Thr Xaa Ser Ile Lys Thr
            900                 905                 910

Leu Ile Arg Ser Val Arg Asp Leu Cys Leu Lys Tyr Glu Ala Lys Gly
        915                 920                 925

Leu Pro Asn Phe Pro Ser Gly Ile Pro Phe Leu Phe Trp Glu Gln Tyr
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Leu Tyr Leu Arg Thr Ser Leu Leu Leu Ala Leu Ala Cys Ala Leu Ala
945                 950                 955                 960

Ala Val Phe Ile Ala Val Met Val Leu Leu Leu Asn Ala Trp Ala Ala
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09/14/2000 15:09 FAX

Val Leu Val Thr Leu Ala Leu Ala Thr Leu Val Leu Gln Leu Leu Gly
980 985 990

Val Met Ala Leu Leu Gly Val Lys Leu Ser Ala Met Pro Ala Val Leu
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Leu Val Leu Ala Ile Gly Arg Gly Val His Phe Thr Val His Leu Cys
1010 1015 1020

Leu Gly Phe Val Thr Ser Ile Gly Cys Lys Arg Arg Arg Ala Ser Leu
1025 1030 1035 1040

Ala Leu Glu Ser Val Leu Ala Pro Val Val His Gly Ala Leu Ala Ala
1045 1050 1055

Ala Leu Ala Ala Ser Met Leu Ala Ala Ser Glu Cys Gly Phe Val Ala
1060 1065 1070

Arg Leu Phe Leu Arg Leu Leu Leu Asp Ile Val Phe Leu Gly Leu Ile
1075 1080 1085

Asp Gly Leu Leu Phe Phe Pro Ile Val Leu Ser Ile Leu Gly Pro Ala
1090 1095 1100

Ala Glu Val Arg Pro Ile Glu His Pro Glu Arg Leu Ser Thr Pro Ser
1105 1110 1115 1120

Pro Lys Cys Ser Pro Ile His Pro Arg Lys Ser Ser Ser Ser Ser Gly
1125 1130 1135

Gly Gly Asp Lys Ser Ser Arg Thr Ser Lys Ser Ala Pro Arg Pro Cys
1140 1145 1150

Ala Pro Ser Leu Thr Thr Ile Thr Glu Glu Pro Ser Ser Trp His Ser
1155 1160 1165

Ser Ala His Ser Val Gln Ser Ser Met Gln Ser Ile Val Val Gln Pro
1170 1175 1180

Glu Val Val Val Glu Thr Thr Thr Tyr Asn Gly Ser Asp Ser Ala Ser
1185 1190 1195 1200

Gly Arg Ser Thr Pro Thr Lys Ser Ser His Gly Gly Ala Ile Thr Thr
1205 1210 1215

Thr Lys Val Thr Ala Thr Ala Asn Ile Lys Val Glu Val Val Thr Pro
1220 1225 1230

Ser Asp Arg Lys Ser Arg Arg Ser Tyr His Tyr Tyr Asp Arg Arg Arg
1235 1240 1245

Asp Arg Asp Glu Asp Arg Asp Arg Asp Arg Glu Arg Asp Arg Asp Arg
1250 1255 1260

Asp Arg Asp Arg Asp Arg Asp Arg Asp Arg Asp Arg Asp Arg Asp Arg
1265 1270 1275 1280

Glu Arg Ser Arg Glu Arg Asp Arg Arg Asp Arg Tyr Arg Asp Glu Arg
1285 1290 1295

Asp His Arg Ala Ser Pro Arg Glu Lys Arg Gln Arg Phe Trp Thr
1300 1305 1310

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(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 4434 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGAAACAAGA GAGCGAGTGA GAGTAGGGAG AGCGTCTGTG TTGTGTGTTG AGTGTCGCCC 60

ACGCACACAG GCGCAAAACA GTGCACACAG ACGCCCGCTG GGCAAGAGAG AGTGAGAGAG 120

AGAAACAGCG GCGCGCGCTC GCCTAATGAA GTTGTTGGCC TGGCTGGCGT GCCGCATCCA 180

CGAGATACAG ATACATCTCT CATGGACCGC GACAGCCTCC CACGCGTTCC GGACACACAC 240

GGCGATGTGG TCGATGAGAA ATTATTCTCG GATCTTTACA TACGCACCAG CTGGGTGGAC 300

GGCCAAGTGG CGCTCGATCA GATAGATAAG GGCAAAGCGC GTGGCAGCCG CACGGCGATC 360

TATCTGCGAT CAGTATTCCA GTCCCACCTC GAAACCCTCG GCAGCTCCGT GCAAAAGCAC 420

GCGGGCAAGG TGCTATTCGT GGCTATCCTG GTGCTGAGCA CCTTCTGCGT CGGCCTGAAG 480

AGCGCCCAGA TCCACTCCAA GGTGCACCAG CTGTGGATCC AGGAGGGCGG CCGGCTGGAG 540

GCGGAACTGG CCTACACACA GAAGACGATC GGCGAGGACG AGTCGGCCAC GCATCAGCTG 600

CTCATTCAGA CGACCCACGA CCCGAACGCC TCCGTCCTGC ATCCGCAGGC GCTGCTTGCC 660

CACCTGGAGG TCCTGGTCAA GGCCACCGCC GTCAAGGTGC ACCTCTACGA CACCGAATGG 720

GGGCTGCGCG ACATGTGCAA CATGCCGAGC ACGCCCTCCT TCGAGGGCAT CTACTACATC 780

GAGCAGATCC TGCGCCACCT CATTCCGTGC TCGATCATCA CGCCGCTGGA CTGTTTCTGG 840

GAGGGAAGCC AGCTGTTGGG TCCGGAATCA GCGGTCGTTA TACCAGGCCT CAACCAACGA 900

CTCCTGTGGA CCACCCTGAA TCCCGCCTCT GTGATGCAGT ATATGAAACA AAAGATGTCC 960

GAGGAAAAGA TCAGCTTCGA CTTCGAGACC GTGGAGCAGT ACATGAAGCG TGCGGCCATT 1020

GGCAGTGGCT ACATGGAGAA GCCCTGCCTG AACCCACTGA ATCCCAATTG CCCGGACACG 1080

GCACCGAACA AGAACAGCAC CCAGCCGCCG GATGTGGGAG CCATCCTGTC CGGAGGCTGC 1140

TACGGTTATG CCGCGAAGCA CATGCACTGG CCGGAGGAGC TGATTGTGGG CGGACGGAAG 1200

AGGAACCGCA GCGGACACTT GAGGAAGGCC CAGGCCCTGC AGTCGGTGGT GCAGCTGATG 1260

ACCGAGAAGG AAATGTACGA CCAGTGGCAG GACAACTACA AGGTGCACCA TCTTGGATGG 1320

ACGCAGGAGA AGGCAGCGGA GGTTTTGAAC GCCTGGCAGC GCAACTTTTC GCGGGAGGTG 1380

GAACAGCTGC TACGTAAACA GTCGAGAATT GCCACCAACT ACGATATCTA CGTGTTCAGC 1440

SUBSTITUTE SHEET (RULE 26)

TCGGCTGCAC TGGATGACAT CCTGGCCAAG TTCTCCCATC CCAGCGCCTT GTCCATTGTC 1500

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GTCCGTGGCC AGAGCAGTGT GGGCGTGGCC GGAGTTCTGC TCATGTGCTT CAGTACCGCC 1620

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GCGGAGAGCA ATCGGCGGGA GCAGACCAAG CTGATTCTCA AGAACGCCAG CACCCAGGTG 1740

GTTCCGTTTT TGGCCCTTGG TCTGGGCGTC GATCACATCT TCATAGTGGG ACCGAGCATC 1800

CTGTTCAGTG CCTGCAGCAC CGCAGGATCC TTCTTTGCGG CCGCCTTTAT TCCGGTGCCG 1860

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CTATTGGTTT TTCCGGCCAT GATTTCGTTG GATCTACGGA GACGTACCGC CGGCAGGGCG 1980

GACATCTTCT GCTGCTGTTT TCCGGTGTGG AAGGAACAGC CGAAGGTGGC ACCTCCGGTG 2040

CTGCCGCTGA ACAACAACAA CGGGCGCGGG GCCCGGCATC CGAAGAGCTG CAACAACAAC 2100

AGGGTGCCGC TGCCCGCCCA GAATCCTCTG CTGGAACAGA GGGCAGACAT CCCTGGGAGC 2160

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TATGCGGTTA CCCAGGGCAA CTTTGAATAT CCCACCCAGC AGCAGTTGCT CAGGGACTAC 2460

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AGTCTGCCAT TGGTCTACGC TCAGATGCCC TTTTACCTCC ACGGACTAAC AGATACCTCG 2940

CAGATCAAGA CCCTGATAGG TCATATTCGC GACCTGAGCG TCAAGTACGA GGGCTTCGGC 3000

CTGCCCAACT ATCCATCGGG CATTCCCTTC ATCTTCTGGG AGCAGTACAT GACCCTGCGC 3060

TCCTCACTGG CCATGATCCT GGCCTGCGTG CTACTCGCCG CCCTGGTGCT GGTCTCCCTG 3120

CTCCTGCTCT CCGTTTGGGC CGCCGTTCTC GTGATCCTCA GCGTTCTGGC CTCGCTGGCC 3180

CAGATCTTTG GGGCCATGAC TCTGCTGGGC ATCAAACTCT CGGCCATTCC GGCAGTCATA 3240

CTCATCCTCA GCGTGGGCAT GATGCTGTGC TTCAATGTGC TGATATCACT GGGCTTCATG 3300

ACATCCGTTG GCAACCGACA GCGCCGCGTC CAGCTGAGCA TGCAGATGTC CCTGGGACCA 3360

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CTTGTCCACG GCATGCTGAC CTCCGGAGTG GCCGTGTTCA TGCTCTCCAC GTCGCCCTTT 3420

GAGTTTGTGA TCCGGCACTT CTGCTGGCTT CTGCTGGTGG TCTTATGCGT TGGCGCCTGC 3480

AACAGCCTTT TGGTGTTCCC CATCCTACTG AGCATGGTGG GACCGGAGGC GGAGCTGGTG 3540

CCGCTGGAGC ATCCAGACCG CATATCCACG CCCTCTCCGC TGCCCGTGCG CAGCAGCAAG 3600

AGATCGGGCA AATCCTATGT GGTGCAGGGA TCGCGATCCT CGCGAGGCAG CTGCCAGAAG 3660

TCGCATCACC ACCACCACAA AGACCTTAAT GATCCATCGC TGACGACGAT CACCGAGGAG 3720

CCGCAGTCGT GGAAGTCCAG CAACTCGTCC ATCCAGATGC CCAATGATTG GACCTACCAG 3780

CCGCGGGAAC AGCGACCCGC CTCCTACGCG GCCCCGCCCC CCGCCTATCA CAAGGCCGCC 3840

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GCCTATCCGC CGGAGCTGCA GAGCATCGTG GTGCAGCCGG AGGTGACGGT GGAGACGACG 3960

CACTCGGACA GCAACACCAC CAAGGTGACG GCCACGGCCA ACATCAAGGT GGAGCTGGCC 4020

ATGCCCGGCA GGGCGGTGCG CAGCTATAAC TTTACGAGTT AGCACTAGCA CTAGTTCCTG 4080

TAGCTATTAG GACGTATCTT TAGACTCTAG CCTAAGCCGT AACCCTATTT GTATCTGTAA 4140

AATCGATTTG TCCAGCGGGT CTGCTGAGGA TTTCGTTCTC ATGGATTCTC ATGGATTCTC 4200

ATGGATGCTT AAATGGCATG GTAATTGGCA AAATATCAAT TTTTGTGTCT CAAAAAGATG 4260

CATTAGCTTA TGGTTTCAAG ATACATTTTT AAAGAGTCCG CCAGATATTT ATATAAAAAA 4320

AATCCAAAAT CGACGTATCC ATGAAAATTG AAAAGCTAAG CAGACCCGTA TGTATGTATA 4380

TGTGTATGCA TGTTAGTTAA TTTCCCGAAG TCCGGTATTT ATAGCAGCTG CCTT 4434

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1285 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Met Asp Arg Asp Ser Leu Pro Arg Val Pro Asp Thr His Gly Asp Val
1 5 10 15

Val Asp Glu Lys Leu Phe Ser Asp Leu Tyr Ile Arg Thr Ser Trp Val
20 25 30

Asp Ala Gln Val Ala Leu Asp Gln Ile Asp Lys Gly Lys Ala Arg Gly
35 40 45

Ser Arg Thr Ala Ile Tyr Leu Arg Ser Val Phe Gln Ser His Leu Glu
50 55 60

Thr Leu Gly Ser Ser Val Gln Lys His Ala Gly Lys Val Leu Phe Val
65 70 75 80

Ala Ile Leu Val Leu Ser Thr Phe Cys Val Gly Leu Lys Ser Ala Gln
85 90 95

Ile His Ser Lys Val His Gln Leu Trp Ile Gln Glu Gly Gly Arg Leu
100 105 110

Glu Ala Glu Leu Ala Tyr Thr Gln Lys Thr Ile Gly Glu Asp Glu Ser
115 120 125

Ala Thr His Gln Leu Leu Ile Gln Thr Thr His Asp Pro Asn Ala Ser
130 135 140

Val Leu His Pro Gln Ala Leu Leu Ala His Leu Glu Val Leu Val Lys
145 150 155 160

Ala Thr Ala Val Lys Val His Leu Tyr Asp Thr Glu Trp Gly Leu Arg
165 170 175

Asp Met Cys Asn Met Pro Ser Thr Pro Ser Phe Glu Gly Ile Tyr Tyr
180 185 190

Ile Glu Gln Ile Leu Arg His Leu Ile Pro Cys Ser Ile Ile Thr Pro
195 200 205

Leu Asp Cys Phe Trp Glu Gly Ser Gln Leu Leu Gly Pro Glu Ser Ala
210 215 220

Val Val Ile Pro Gly Leu Asn Gln Arg Leu Leu Trp Thr Thr Leu Asn
225 230 235 240

Pro Ala Ser Val Met Gln Tyr Met Lys Gln Lys Met Ser Glu Glu Lys
245 250 255

Ile Ser Phe Asp Phe Glu Thr Val Glu Gln Tyr Met Lys Arg Ala Ala
260 265 270

Ile Gly Ser Gly Tyr Met Glu Lys Pro Cys Leu Asn Pro Leu Asn Pro
275 280 285

Asn Cys Pro Asp Thr Ala Pro Asn Lys Asn Ser Thr Gln Pro Pro Asp
290 295 300

Val Gly Ala Ile Leu Ser Gly Gly Cys Tyr Gly Tyr Ala Ala Lys His
305 310 315 320

Met His Trp Pro Glu Glu Leu Ile Val Gly Gly Arg Lys Arg Asn Arg
325 330 335

Ser Gly His Leu Arg Lys Ala Gln Ala Leu Gln Ser Val Val Gln Leu
340 345 350

Met Thr Glu Lys Glu Met Tyr Asp Gln Trp Gln Asp Asn Tyr Lys Val
355 360 365

His His Leu Gly Trp Thr Gln Glu Lys Ala Ala Glu Val Leu Asn Ala
370 375 380

Trp Gln Arg Asn Phe Ser Arg Glu Val Glu Gln Leu Leu Arg Lys Gln
385 390 395 400

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Ser Arg Ile Ala Thr Asn Tyr Asp Ile Tyr Val Phe Ser Ser Ala Ala
405 410 415

Leu Asp Asp Ile Leu Ala Lys Phe Ser His Pro Ser Ala Leu Ser Ile
420 425 430

Val Ile Gly Val Ala Val Thr Val Leu Tyr Ala Phe Cys Thr Leu Leu
435 440 445

Arg Trp Arg Asp Pro Val Arg Gly Gln Ser Ser Val Gly Val Ala Gly
450 455 460

Val Leu Leu Met Cys Phe Ser Thr Ala Ala Gly Leu Gly Leu Ser Ala
465 470 475 480

Leu Leu Gly Ile Val Phe Asn Ala Leu Thr Ala Ala Tyr Ala Glu Ser
485 490 495

Asn Arg Arg Glu Gln Thr Lys Leu Ile Leu Lys Asn Ala Ser Thr Gln
500 505 510

Val Val Pro Phe Leu Ala Leu Gly Leu Gly Val Asp His Ile Phe Ile
515 520 525

Val Gly Pro Ser Ile Leu Phe Ser Ala Cys Ser Thr Ala Gly Ser Phe
530 535 540

Phe Ala Ala Ala Phe Ile Pro Val Pro Ala Leu Lys Val Phe Cys Leu
545 550 555 560

Gln Ala Ala Ile Val Met Cys Ser Asn Leu Ala Ala Ala Leu Leu Val
565 570 575

Phe Pro Ala Met Ile Ser Leu Asp Leu Arg Arg Arg Thr Ala Gly Arg
580 585 590

Ala Asp Ile Phe Cys Cys Cys Phe Pro Val Trp Lys Glu Gln Pro Lys
595 600 605

Val Ala Pro Pro Val Leu Pro Leu Asn Asn Asn Asn Gly Arg Gly Ala
610 615 620

Arg His Pro Lys Ser Cys Asn Asn Asn Arg Val Pro Leu Pro Ala Gln
625 630 635 640

Asn Pro Leu Leu Glu Gln Arg Ala Asp Ile Pro Gly Ser Ser His Ser
645 650 655

Leu Ala Ser Phe Ser Leu Ala Thr Phe Ala Phe Gln His Tyr Thr Pro
660 665 670

Phe Leu Met Arg Ser Trp Val Lys Phe Leu Thr Val Met Gly Phe Leu
675 680 685

Ala Ala Leu Ile Ser Ser Leu Tyr Ala Ser Thr Arg Leu Gln Asp Gly
690 695 700

Leu Asp Ile Ile Asp Leu Val Pro Lys Asp Ser Asn Glu His Lys Phe
705 710 715 720

Leu Asp Ala Gln Thr Arg Leu Phe Gly Phe Tyr Ser Met Tyr Ala Val
725 730 735

Thr Gln Gly Asn Phe Glu Tyr Pro Thr Gln Gln Gln Leu Leu Arg Asp

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740 745 750

Tyr His Asp Ser Phe Arg Val Pro His Val Ile Lys Asn Asp Asn Gly
755 760 765

Gly Leu Pro Asp Phe Trp Leu Leu Leu Phe Ser Glu Trp Leu Gly Asn
770 775 780

Leu Gln Lys Ile Phe Asp Glu Glu Tyr Arg Asp Gly Arg Leu Thr Lys
785 790 795 800

Glu Cys Trp Phe Pro Asn Ala Ser Ser Asp Ala Ile Leu Ala Tyr Lys
805 810 815

Leu Ile Val Gln Thr Gly His Val Asp Asn Pro Val Asp Lys Glu Leu
820 825 830

Val Leu Thr Asn Arg Leu Val Asn Ser Asp Gly Ile Ile Asn Gln Arg
835 840 845

Ala Phe Tyr Asn Tyr Leu Ser Ala Trp Ala Thr Asn Asp Val Phe Ala
850 855 860

Tyr Gly Ala Ser Gln Gly Lys Leu Tyr Pro Glu Pro Arg Gln Tyr Phe
865 870 875 880

His Gln Pro Asn Glu Tyr Asp Leu Lys Ile Pro Lys Ser Leu Pro Leu
885 890 895

Val Tyr Ala Gln Met Pro Phe Tyr Leu His Gly Leu Thr Asp Thr Ser
900 905 910

Gln Ile Lys Thr Leu Ile Gly His Ile Arg Asp Leu Ser Val Lys Tyr
915 920 925

Glu Gly Phe Gly Leu Pro Asn Tyr Pro Ser Gly Ile Pro Phe Ile Phe
930 935 940

Trp Glu Gln Tyr Met Thr Leu Arg Ser Ser Leu Ala Met Ile Leu Ala
945 950 955 960

Cys Val Leu Leu Ala Ala Leu Val Leu Val Ser Leu Leu Leu Leu Ser
965 970 975

Val Trp Ala Ala Val Leu Val Ile Leu Ser Val Leu Ala Ser Leu Ala
980 985 990

Gln Ile Phe Gly Ala Met Thr Leu Leu Gly Ile Lys Leu Ser Ala Ile
995 1000 1005

Pro Ala Val Ile Leu Ile Leu Ser Val Gly Met Met Leu Cys Phe Asn
1010 1015 1020

Val Leu Ile Ser Leu Gly Phe Met Thr Ser Val Gly Asn Arg Gln Arg
1025 1030 1035 1040

Arg Val Gln Leu Ser Met Gln Met Ser Leu Gly Pro Leu Val His Gly
1045 1050 1055

Met Leu Thr Ser Gly Val Ala Val Phe Met Leu Ser Thr Ser Pro Phe
1060 1065 1070

Glu Phe Val Ile Arg His Phe Cys Trp Leu Leu Leu Val Val Leu Cys

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        1075                1080                1085

Val Gly Ala Cys Asn Ser Leu Leu Val Phe Pro Ile Leu Leu Ser Met
    1090                1095                1100

Val Gly Pro Glu Ala Glu Leu Val Pro Leu Glu His Pro Asp Arg Ile
1105                1110                1115                1120

Ser Thr Pro Ser Pro Leu Pro Val Arg Ser Ser Lys Arg Ser Gly Lys
                1125                1130                1135

Ser Tyr Val Val Gln Gly Ser Arg Ser Ser Arg Gly Ser Cys Gln Lys
            1140                1145                1150

Ser His His His His His Lys Asp Leu Asn Asp Pro Ser Leu Thr Thr
        1155                1160                1165

Ile Thr Glu Glu Pro Gln Ser Trp Lys Ser Ser Asn Ser Ser Ile Gln
    1170                1175                1180

Met Pro Asn Asp Trp Thr Tyr Gln Pro Arg Glu Gln Arg Pro Ala Ser
1185                1190                1195                1200

Tyr Ala Ala Pro Pro Pro Ala Tyr His Lys Ala Ala Ala Gln Gln His
                1205                1210                1215

His Gln His Gln Gly Pro Pro Thr Thr Pro Pro Pro Pro Phe Pro Thr
            1220                1225                1230

Ala Tyr Pro Pro Glu Leu Gln Ser Ile Val Val Gln Pro Glu Val Thr
        1235                1240                1245

Val Glu Thr Thr His Ser Asp Ser Asn Thr Thr Lys Val Thr Ala Thr
    1250                1255                1260

Ala Asn Ile Lys Val Glu Leu Ala Met Pro Gly Arg Ala Val Arg Ser
1265                1270                1275                1280

Tyr Asn Phe Thr Ser
                1285
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 345 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

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AAGGTCCATC AGCTTTGGAT ACAGGAAGGT GGTTCGCTCG AGCATGAGCT AGCCTACACG      60

CAGAAATCGC TCGGCGAGAT GGACTCCTCC ACGCACCAGC TGCTAATCCA AACNCCCAAA     120

GATATGGACG CCTCGATACT GCACCCGAAC GCGCTACTGA CGCACCTGGA CGTGGTGAAG     180

AAAGCGATCT CGGTGACGGT GCACATGTAC GACATCACGT GGAGNCTCAA GGACATGTGC     240
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TACTCGCCCA GCATACCGAG NTTCGATACG CACTTTATCG AGCAGATCTT CGAGAACATC 300

ATACCGTGCG CGATCATCAC GCCGCTGGAT TGCTTTTGGG AGGGA 345

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 115 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

Lys Val His Gln Leu Trp Ile Gln Glu Gly Gly Ser Leu Glu His Glu
1 5 10 15

Leu Ala Tyr Thr Gln Lys Ser Leu Gly Glu Met Asp Ser Ser Thr His
20 25 30

Gln Leu Leu Ile Gln Thr Pro Lys Asp Met Asp Ala Ser Ile Leu His
35 40 45

Pro Asn Ala Leu Leu Thr His Leu Asp Val Val Lys Lys Ala Ile Ser
50 55 60

Val Thr Val His Met Tyr Asp Ile Thr Trp Xaa Leu Lys Asp Met Cys
65 70 75 80

Tyr Ser Pro Ser Ile Pro Xaa Phe Asp Thr His Phe Ile Glu Gln Ile
85 90 95

Phe Glu Asn Ile Ile Pro Cys Ala Ile Ile Thr Pro Leu Asp Cys Phe
100 105 110

Trp Glu Gly
115

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5187 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

GGGTCTGTCA CCCGGAGCCG GAGTCCCCGG CGGCCAGCAG CGTCCTCGCG AGCCGAGCGC 60

CCAGGCGCGC CCGGAGCCCG CGGCGGCGGC GGCAACATGG CCTCGGCTGG TAACGCCGCC 120

GGGGCCCTGG GCAGGCAGGC CGGCGGCGGG AGGCGCAGAC GGACCGGGGG ACCGCACCGC 180

GCCGCGCCGG ACCGGGACTA TCTGCACCGG CCCAGCTACT GCGACGCCGC CTTCGCTCTG 240

GAGCAGATTT CCAAGGGGAA GGCTACTGGC CGGAAAGCGC CGCTGTGGCT GAGAGCGAAG 300

TTTCAGAGAC TCTTATTTAA ACTGGGTTGT TACATTCAAA AGAACTGCGG CAAGTTTTTG 360

GTTGTGGGTC TCCTCATATT TGGGGCCTTC GCTGTGGGAT TAAAGGCAGC TAATCTCGAG 420

ACCAACGTGG AGGAGCTGTG GGTGGAAGTT GGTGGACGAG TGAGTCGAGA ATTAAATTAT 480

ACCCGTCAGA AGATAGGAGA AGAGGCTATG TTTAATCCTC AACTCATGAT ACAGACTCCA 540

AAAGAAGAAG GCGCTAATGT TCTGACCACA GAGGCTCTCC TGCAACACCT GGACTCAGCA 600

CTCCAGGCCA GTCGTGTGCA CGTCTACATG TATAACAGGC AATGGAAGTT GGAACATTTG 660

TGCTACAAAT CAGGGGAACT TATCACGGAG ACAGGTTACA TGGATCAGAT AATAGAATAC 720

CTTTACCCTT GCTTAATCAT TACACCTTTG GACTGCTTCT GGGAAGGGGC AAAGCTACAG 780

TCCGGGACAG CATACCTCCT AGGTAAGCCT CCTTTACGGT GGACAAACTT TGACCCCTTG 840

GAATTCCTAG AAGAGTTAAA GAAAATAAAC TACCAAGTGG ACAGCTGGGA GGAAATGCTG 900

AATAAAGCCG AAGTTGGCCA TGGGTACATG GACCGGCCTT GCCTCAACCC AGCCGACCCA 960

GATTGCCCTG CCACAGCCCC TAACAAAAAT TCAACCAAAC CTCTTGATGT GGCCCTTGTT 1020

TTGAATGGTG GATGTCAAGG TTTATCCAGG AAGTATATGC ATTGGCAGGA GGAGTTGATT 1080

GTGGGTGGTA CCGTCAAGAA TGCCACTGGA AAACTTGTCA GCGCTCACGC CCTGCAAACC 1140

ATGTTCCAGT TAATGACTCC CAAGCAAATG TATGAACACT TCAGGGGCTA CGACTATGTC 1200

TCTCACATCA ACTGGAATGA AGACAGGGCA GCCGCCATCC TGGAGGCCTG GCAGAGGACT 1260

TACGTGGAGG TGGTTCATCA AAGTGTCGCC CCAAACTCCA CTCAAAAGGT GCTTCCCTTC 1320

ACAACCACGA CCCTGGACGA CATCCTAAAA TCCTTCTCTG ATGTCAGTGT CATCCGAGTG 1380

GCCAGCGGCT ACCTACTGAT GCTTGCCTAT GCCTGTTTAA CCATGCTGCG CTGGGACTGC 1440

TCCAAGTCCC AGGGTGCCGT GGGGCTGGCT GGCGTCCTGT TGGTTGCGCT GTCAGTGGCT 1500

GCAGGATTGG GCCTCTGCTC CTTGATTGGC ATTTCTTTTA ATGCTGCGAC AACTCAGGTT 1560

TTGCCGTTTC TTGCTCTTGG TGTTGGTGTG GATGATGTCT TCCTCCTGGC CCATGCATTC 1620

AGTGAAACAG GACAGAATAA GAGGATTCCA TTTGAGGACA GGACTGGGGA GTGCCTCAAG 1680

CGCACCGGAG CCAGCGTGGC CCTCACCTCC ATCAGCAATG TCACCGCCTT CTTCATGGCC 1740

GCATTGATCC CTATCCCTGC CCTGCGAGCG TTCTCCCTCC AGGCTGCTGT GGTGGTGGTA 1800

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CGTGAGGACA GAAGATTGGA TATTTTCTGC TGTTTCACAA GCCCCTGTGT CAGCAGGGTG 1920

ATTCAAGTTG AGCCACAGGC CTACACAGAG CCTCACAGTA ACACCCGGTA CAGCCCCCCA 1980

CCCCCATACA CCAGCCACAG CTTCGCCCAC GAAACCCATA TCACTATGCA GTCCACCGTT 2040

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CAGCTCCGCA CAGAGTATGA CCCTCACACG CACGTGTACT ACACCACCGC CGAGCCACGC 2100

TCTGAGATCT CTGTACAGCC TGTTACCGTC ACCCAGGACA ACCTCAGCTG TCAGAGTCCC 2160

GAGAGCACCA GCTCTACCAG GGACCTGCTC TCCCAGTTCT CAGACTCCAG CCTCCACTGC 2220

CTCGAGCCCC CCTGCACCAA GTGGACACTC TCTTCGTTTG CAGAGAAGCA CTATGCTCCT 2280

TTCCTCCTGA AACCCAAAGC CAAGGTTGTG GTAATCCTTC TTTTCCTGGG CTTGCTGGGG 2340

GTCAGCCTTT ATGGGACCAC CCGAGTGAGA GACGGGCTGG ACCTCACGGA CATTGTTCCC 2400

CGGGAAACCA GAGAATATGA CTTCATAGCT GCCCAGTTCA AGTACTTCTC TTTCTACAAC 2460

ATGTATATAG TCACCCAGAA AGCAGACTAC CCGAATATCC AGCACCTACT TTACGACCTT 2520

CATAAGAGTT TCAGCAATGT GAAGTATGTC ATGCTGGAGG AGAACAAGCA ACTTCCCCAA 2580

ATGTGGCTGC ACTACTTTAG AGACTGGCTT CAAGGACTTC AGGATGCATT TGACAGTGAC 2640

TGGGAAACTG GGAGGATCAT GCCAAACAAT TATAAAAATG GATCAGATGA CGGGGTCCTC 2700

GCTTACAAAC TCCTGGTGCA GACTGGCAGC CGAGACAAGC CCATCGACAT TAGTCAGTTG 2760

ACTAAACAGC GTCTGGTAGA CGCAGATGGC ATCATTAATC CGAGCGCTTT CTACATCTAC 2820

CTGACCGCTT GGGTCAGCAA CGACCCTGTA GCTTACGCTG CCTCCCAGGC CAACATCCGG 2880

CCTCACCGGC CGGAGTGGGT CCATGACAAA GCCGACTACA TGCCAGAGAC CAGGCTGAGA 2940

ATCCCAGCAG CAGAGCCCAT CGAGTACGCT CAGTTCCCTT TCTACCTCAA CGGCCTACGA 3000

GACACCTCAG ACTTTGTGGA AGCCATAGAA AAAGTGAGAG TCATCTGTAA CAACTATACG 3060

AGCCTGGGAC TGTCCAGCTA CCCCAATGGC TACCCCTTCC TGTTCTGGGA GCAATACATC 3120

AGCCTGCGCC ACTGGCTGCT GCTATCCATC AGCGTGGTGC TGGCCTGCAC GTTTCTAGTG 3180

TGCGCAGTCT TCCTCCTGAA CCCCTGGACG GCCGGGATCA TTGTCATGGT CCTGGCTCTG 3240

ATGACCGTTG AGCTCTTTGG CATGATGGGC CTCATTGGGA TCAAGCTGAG TGCTGTGCCT 3300

GTGGTCATCC TGATTGCATC TGTTGGCATC GGAGTGGAGT TCACCGTCCA CGTGGCTTTG 3360

GCCTTTCTGA CAGCCATTGG GGACAAGAAC CACAGGGCTA TGCTCGCTCT GGAACACATG 3420

TTTGCTCCCG TTCTGGACGG TGCTGTGTCC ACTCTGCTGG GTGTACTGAT GCTTGCAGGG 3480

TCCGAATTTG ATTTCATTGT CAGATACTTC TTTGCCGTCC TGGCCATTCT CACCGTCTTG 3540

GGGGTTCTCA ATGGACTGGT TCTGCTGCCT GTCCTCTTAT CCTTCTTTGG ACCGTGTCCT 3600

GAGGTGTCTC CAGCCAATGG CCTAAACCGA CTGCCCACTC CTTCGCCTGA GCCGCCTCCA 3660

AGTGTCGTCC GGTTTGCCGT GCCTCCTGGT CACACGAACA ATGGGTCTGA TTCCTCCGAC 3720

TCGGAGTACA GCTCTCAGAC CACGGTGTCT GGCATCAGTG AGGAGCTCAG GCAATACGAA 3780

GCACAGCAGG GTGCCGGAGG CCCTGCCCAC CAAGTGATTG TGGAAGCCAC AGAAAACCCT 3840

GTCTTTGCCC GGTCCACTGT GGTCCATCCG GACTCCAGAC ATCAGCCTCC CTTGACCCCT 3900

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CGGCAACAGC CCCACCTGGA CTCTGGCTCC TTGTCCCCTG GACGGCAAGG CCAGCAGCCT 3960

CGAAGGGATC CCCCTAGAGA AGGCTTGCGG CCACCCCCCT ACAGACCGCG CAGAGACGCT 4020

TTTGAAATTT CTACTGAAGG GCATTCTGGC CCTAGCAATA GGGACCGCTC AGGGCCCCGT 4080

GGGGCCCGTT CTCACAACCC TCGGAACCCA ACGTCCACCG CCATGGGCAG CTCTGTGCCC 4140

AGCTACTGCC AGCCCATCAC CACTGTGACG GCTTCTGCTT CGGTGACTGT TGCTGTGCAT 4200

CCCCCGCCTG GACCTGGGCG CAACCCCCGA GGGGGGCCCT GTCCAGGCTA TGAGAGCTAC 4260

CCTGAGACTG ATCACGGGGT ATTTGAGGAT CCTCATGTGC CTTTTCATGT CAGGTGTGAG 4320

AGGAGGGACT CAAAGGTGGA GGTCATAGAG CTACAGGACG TGGAATGTGA GGAGAGGCCG 4380

TGGGGGAGCA GCTCCAACTG AGGGTAATTA AAATCTGAAG CAAAGAGGCC AAAGATTGGA 4440

AAGCCCCGCC CCCACCTCTT TCCAGAACTG CTTGAAGAGA ACTGCTTGGA ATTATGGGAA 4500

GGCAGTTCAT TGTTACTGTA ACTGATTGTA TTATTKKGTG AAATATTTCT ATAAATATTT 4560

AARAGGTGTA CACATGTAAT ATACATGGAA ATGCTGTACA GTCTATTTCC TGGGGCCTCT 4620

CCACTCCTGC CCCAGAGTGG GGAGACCACA GGGGCCCTTT CCCCTGTGTA CATTGGTCTC 4680

TGTGCCACAA CCAAGCTTAA CTTAGTTTTA AAAAAAATCT CCCAGCATAT GTCGCTGCTG 4740

CTTAAATATT GTATAATTTA CTTGTATAAT TCTATGCAAA TATTGCTTAT GTAATAGGAT 4800

TATTTGTAAA GGTTTCTGTT TAAAATATTT TAAATTTGCA TATCACAACC CTGTGGTAGG 4860

ATGAATTGTT ACTGTTAACT TTTGAACACG CTATGCGTGG TAATTGTTTA ACGAGCAGAC 4920

ATGAAGAAAA CAGGTTAATC CCAGTGGCTT CTCTAGGGGT AGTTGTATAT GGTTCGCATG 4980

GGTGGATGTG TGTGTGCATG TGACTTTCCA ATGTACTGTA TTGTGGTTTG TTGTTGTTGT 5040

TGCTGTTGTT GTTCATTTTG GTGTTTTTGG TTGCTTTGTA TGATCTTAGC TCTGGCCTAG 5100

GTGGGCTGGG AAGGTCCAGG TCTTTTTCTG TCGTGATGCT GGTGGAAAGG TGACCCCAAT 5160

CATCTGTCCT ATTCTCTGGG ACTATTC 5187

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1434 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met Ala Ser Ala Gly Asn Ala Ala Gly Ala Leu Gly Arg Gln Ala Gly
1 5 10 15

Gly Gly Arg Arg Arg Arg Thr Gly Gly Pro His Arg Ala Ala Pro Asp

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                20                  25                  30

Arg Asp Tyr Leu His Arg Pro Ser Tyr Cys Asp Ala Ala Phe Ala Leu
        35                  40                  45

Glu Gln Ile Ser Lys Gly Lys Ala Thr Gly Arg Lys Ala Pro Leu Trp
    50                  55                  60

Leu Arg Ala Lys Phe Gln Arg Leu Leu Phe Lys Leu Gly Cys Tyr Ile
65                  70                  75                  80

Gln Lys Asn Cys Gly Lys Phe Leu Val Val Gly Leu Leu Ile Phe Gly
                85                  90                  95

Ala Phe Ala Val Gly Leu Lys Ala Ala Asn Leu Glu Thr Asn Val Glu
            100                 105                 110

Glu Leu Trp Val Glu Val Gly Gly Arg Val Ser Arg Glu Leu Asn Tyr
        115                 120                 125
Thr Arg Gln Lys Ile Gly Glu Glu Ala Met Phe Asn Pro Gln Leu Met
    130                 135                 140

Ile Gln Thr Pro Lys Glu Glu Gly Ala Asn Val Leu Thr Thr Glu Ala
145                 150                 155                 160

Leu Leu Gln His Leu Asp Ser Ala Leu Gln Ala Ser Arg Val His Val
                165                 170                 175

Tyr Met Tyr Asn Arg Gln Trp Lys Leu Glu His Leu Cys Tyr Lys Ser
            180                 185                 190

Gly Glu Leu Ile Thr Glu Thr Gly Tyr Met Asp Gln Ile Ile Glu Tyr
        195                 200                 205

Leu Tyr Pro Cys Leu Ile Ile Thr Pro Leu Asp Cys Phe Trp Glu Gly
    210                 215                 220

Ala Lys Leu Gln Ser Gly Thr Ala Tyr Leu Leu Gly Lys Pro Pro Leu
225                 230                 235                 240

Arg Trp Thr Asn Phe Asp Pro Leu Glu Phe Leu Glu Glu Leu Lys Lys
                245                 250                 255

Ile Asn Tyr Gln Val Asp Ser Trp Glu Glu Met Leu Asn Lys Ala Glu
            260                 265                 270

Val Gly His Gly Tyr Met Asp Arg Pro Cys Leu Asn Pro Ala Asp Pro
        275                 280                 285

Asp Cys Pro Ala Thr Ala Pro Asn Lys Asn Ser Thr Lys Pro Leu Asp
    290                 295                 300

Val Ala Leu Val Leu Asn Gly Gly Cys Gln Gly Leu Ser Arg Lys Tyr
305                 310                 315                 320

Met His Trp Gln Glu Glu Leu Ile Val Gly Gly Thr Val Lys Asn Ala
                325                 330                 335

Thr Gly Lys Leu Val Ser Ala His Ala Leu Gln Thr Met Phe Gln Leu
            340                 345                 350

Met Thr Pro Lys Gln Met Tyr Glu His Phe Arg Gly Tyr Asp Tyr Val
        355                 360                 365
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Substitute Sheet (Rule 26)

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Ser His Ile Asn Trp Asn Glu Asp Arg Ala Ala Ala Ile Leu Glu Ala
    370                 375                 380

Trp Gln Arg Thr Tyr Val Glu Val Val His Gln Ser Val Ala Pro Asn
385                 390                 395                 400

Ser Thr Gln Lys Val Leu Pro Phe Thr Thr Thr Thr Leu Asp Asp Ile
                405                 410                 415

Leu Lys Ser Phe Ser Asp Val Ser Val Ile Arg Val Ala Ser Gly Tyr
            420                 425                 430

Leu Leu Met Leu Ala Tyr Ala Cys Leu Thr Met Leu Arg Trp Asp Cys
        435                 440                 445

Ser Lys Ser Gln Gly Ala Val Gly Leu Ala Gly Val Leu Leu Val Ala
    450                 455                 460

Leu Ser Val Ala Ala Gly Leu Gly Leu Cys Ser Leu Ile Gly Ile Ser
465                 470                 475                 480

Phe Asn Ala Ala Thr Thr Gln Val Leu Pro Phe Leu Ala Leu Gly Val
                485                 490                 495

Gly Val Asp Asp Val Phe Leu Leu Ala His Ala Phe Ser Glu Thr Gly
            500                 505                 510

Gln Asn Lys Arg Ile Pro Phe Glu Asp Arg Thr Gly Glu Cys Leu Lys
        515                 520                 525

Arg Thr Gly Ala Ser Val Ala Leu Thr Ser Ile Ser Asn Val Thr Ala
    530                 535                 540

Phe Phe Met Ala Ala Leu Ile Pro Ile Pro Ala Leu Arg Ala Phe Ser
545                 550                 555                 560

Leu Gln Ala Ala Val Val Val Val Phe Asn Phe Ala Met Val Leu Leu
                565                 570                 575

Ile Phe Pro Ala Ile Leu Ser Met Asp Leu Tyr Arg Arg Glu Asp Arg
            580                 585                 590

Arg Leu Asp Ile Phe Cys Cys Phe Thr Ser Pro Cys Val Ser Arg Val
        595                 600                 605

Ile Gln Val Glu Pro Gln Ala Tyr Thr Glu Pro His Ser Asn Thr Arg
    610                 615                 620

Tyr Ser Pro Pro Pro Pro Tyr Thr Ser His Ser Phe Ala His Glu Thr
625                 630                 635                 640

His Ile Thr Met Gln Ser Thr Val Gln Leu Arg Thr Glu Tyr Asp Pro
                645                 650                 655

His Thr His Val Tyr Tyr Thr Thr Ala Glu Pro Arg Ser Glu Ile Ser
            660                 665                 670

Val Gln Pro Val Thr Val Thr Gln Asp Asn Leu Ser Cys Gln Ser Pro
        675                 680                 685

Glu Ser Thr Ser Ser Thr Arg Asp Leu Leu Ser Gln Phe Ser Asp Ser
    690                 695                 700
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Ser Leu His Cys Leu Glu Pro Pro Cys Thr Lys Trp Thr Leu Ser Ser
705                 710                 715                 720

Phe Ala Glu Lys His Tyr Ala Pro Phe Leu Leu Lys Pro Lys Ala Lys
                725                 730                 735

Val Val Val Ile Leu Leu Phe Leu Gly Leu Leu Gly Val Ser Leu Tyr
            740                 745                 750
Gly Thr Thr Arg Val Arg Asp Gly Leu Asp Leu Thr Asp Ile Val Pro
        755                 760                 765

Arg Glu Thr Arg Glu Tyr Asp Phe Ile Ala Ala Gln Phe Lys Tyr Phe
    770                 775                 780

Ser Phe Tyr Asn Met Tyr Ile Val Thr Gln Lys Ala Asp Tyr Pro Asn
785                 790                 795                 800

Ile Gln His Leu Leu Tyr Asp Leu His Lys Ser Phe Ser Asn Val Lys
                805                 810                 815

Tyr Val Met Leu Glu Glu Asn Lys Gln Leu Pro Gln Met Trp Leu His
            820                 825                 830

Tyr Phe Arg Asp Trp Leu Gln Gly Leu Gln Asp Ala Phe Asp Ser Asp
        835                 840                 845

Trp Glu Thr Gly Arg Ile Met Pro Asn Asn Tyr Lys Asn Gly Ser Asp
    850                 855                 860

Asp Gly Val Leu Ala Tyr Lys Leu Leu Val Gln Thr Gly Ser Arg Asp
865                 870                 875                 880

Lys Pro Ile Asp Ile Ser Gln Leu Thr Lys Gln Arg Leu Val Asp Ala
                885                 890                 895

Asp Gly Ile Ile Asn Pro Ser Ala Phe Tyr Ile Tyr Leu Thr Ala Trp
            900                 905                 910

Val Ser Asn Asp Pro Val Ala Tyr Ala Ala Ser Gln Ala Asn Ile Arg
        915                 920                 925

Pro His Arg Pro Glu Trp Val His Asp Lys Ala Asp Tyr Met Pro Glu
    930                 935                 940

Thr Arg Leu Arg Ile Pro Ala Ala Glu Pro Ile Glu Tyr Ala Gln Phe
945                 950                 955                 960

Pro Phe Tyr Leu Asn Gly Leu Arg Asp Thr Ser Asp Phe Val Glu Ala
                965                 970                 975

Ile Glu Lys Val Arg Val Ile Cys Asn Asn Tyr Thr Ser Leu Gly Leu
            980                 985                 990

Ser Ser Tyr Pro Asn Gly Tyr Pro Phe Leu Phe Trp Glu Gln Tyr Ile
        995                 1000                1005

Ser Leu Arg His Trp Leu Leu Leu Ser Ile Ser Val Val Leu Ala Cys
    1010                1015                1020

Thr Phe Leu Val Cys Ala Val Phe Leu Leu Asn Pro Trp Thr Ala Gly
1025                1030                1035                1040
```

Substitute Sheet (Rule 26)

Ile Ile Val Met Val Leu Ala Leu Met Thr Val Glu Leu Phe Gly Met
1045 1050 1055

Met Gly Leu Ile Gly Ile Lys Leu Ser Ala Val Pro Val Val Ile Leu
1060 1065 1070

Ile Ala Ser Val Gly Ile Gly Val Glu Phe Thr Val His Val Ala Leu
1075 1080 1085

Ala Phe Leu Thr Ala Ile Gly Asp Lys Asn His Arg Ala Met Leu Ala
1090 1095 1100

Leu Glu His Met Phe Ala Pro Val Leu Asp Gly Ala Val Ser Thr Leu
1105 1110 1115 1120

Leu Gly Val Leu Met Leu Ala Gly Ser Glu Phe Asp Phe Ile Val Arg
1125 1130 1135

Tyr Phe Phe Ala Val Leu Ala Ile Leu Thr Val Leu Gly Val Leu Asn
1140 1145 1150

Gly Leu Val Leu Leu Pro Val Leu Leu Ser Phe Phe Gly Pro Cys Pro
1155 1160 1165

Glu Val Ser Pro Ala Asn Gly Leu Asn Arg Leu Pro Thr Pro Ser Pro
1170 1175 1180

Glu Pro Pro Pro Ser Val Val Arg Phe Ala Val Pro Pro Gly His Thr
1185 1190 1195 1200

Asn Asn Gly Ser Asp Ser Ser Asp Ser Glu Tyr Ser Ser Gln Thr Thr
1205 1210 1215

Val Ser Gly Ile Ser Glu Glu Leu Arg Gln Tyr Glu Ala Gln Gln Gly
1220 1225 1230

Ala Gly Gly Pro Ala His Gln Val Ile Val Glu Ala Thr Glu Asn Pro
1235 1240 1245

Val Phe Ala Arg Ser Thr Val Val His Pro Asp Ser Arg His Gln Pro
1250 1255 1260

Pro Leu Thr Pro Arg Gln Gln Pro His Leu Asp Ser Gly Ser Leu Ser
1265 1270 1275 1280

Pro Gly Arg Gln Gly Gln Gln Pro Arg Arg Asp Pro Pro Arg Glu Gly
1285 1290 1295

Leu Arg Pro Pro Pro Tyr Arg Pro Arg Arg Asp Ala Phe Glu Ile Ser
1300 1305 1310

Thr Glu Gly His Ser Gly Pro Ser Asn Arg Asp Arg Ser Gly Pro Arg
1315 1320 1325

Gly Ala Arg Ser His Asn Pro Arg Asn Pro Thr Ser Thr Ala Met Gly
1330 1335 1340

Ser Ser Val Pro Ser Tyr Cys Gln Pro Ile Thr Thr Val Thr Ala Ser
1345 1350 1355 1360

Ala Ser Val Thr Val Ala Val His Pro Pro Pro Gly Pro Gly Arg Asn
1365 1370 1375

Pro Arg Gly Gly Pro Cys Pro Gly Tyr Glu Ser Tyr Pro Glu Thr Asp
1380 1385 1390

His Gly Val Phe Glu Asp Pro His Val Pro Phe His Val Arg Cys Glu
1395 1400 1405

Arg Arg Asp Ser Lys Val Glu Val Ile Glu Leu Gln Asp Val Glu Cys
1410 1415 1420

Glu Glu Arg Pro Trp Gly Ser Ser Ser Asn
1425 1430

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ile Ile Thr Pro Leu Asp Cys Phe Trp Glu Gly
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Leu Ile Val Gly Gly
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 7 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Pro Phe Phe Trp Glu Gln Tyr

1 5

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 28 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GGACGAATTC AARGTNCAYC ARYTNTGG 28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGACGAATTC CYTCCCARAA RCANTC 26

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:
GGACGAATTC YTNGANTGYT TYTGGGA 27

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CATACCAGCC AAGCTTGTCN GGCCARTGCA T 31

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 5288 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAATTCCGGG GACCGCAAGG AGTGCCGCGG AAGCGCCCGA AGGACAGGCT CGCTCGGCGC 60

GCCGGCTCTC GCTCTTCCGC GAACTGGATG TGGGCAGCGG CGGCCGCAGA GACCTCGGGA 120

CCCCCGCGCA ATGTGGCAAT GGAAGGCGCA GGGTCTGACT CCCCGGCAGC GGCCGCGGCC 180

GCAGCGGCAG CAGCGCCCGC CGTGTGAGCA GCAGCAGCGG CTGGTCTGTC AACCGGAGCC 240

CGAGCCCGAG CAGCCTGCGG CCAGCAGCGT CCTCGCAAGC CGAGCGCCCA GGCGCGCCAG 300

GAGCCCGCAG CAGCGGCAGC AGCGCGCCGG GCCGCCCGGG AAGCCTCCGT CCCCGCGGCG 360

GCGGCGGCGG CGGCGGCGGC AACATGGCCT CGGCTGGTAA CGCCGCCGAG CCCCAGGACC 420

GCGGCGGCGG CGGCAGCGGC TGTATCGGTG CCCCGGGACG GCCGGCTGGA GGCGGGAGGC 480

GCAGACGGAC GGGGGGGCTG CGCCGTGCTG CCGCGCCGGA CCGGGACTAT CTGCACCGGC 540

CCAGCTACTG CGACGCCGCC TTCGCTCTGG AGCAGATTTC CAAGGGGAAG GCTACTGGCC 600

GGAAAGCGCC ACTGTGGCTG AGAGCGAAGT TTCAGAGACT CTTATTTAAA CTGGGTTGTT 660

ACATTCAAAA AAACTGCGGC AAGTTCTTGG TTGTGGGCCT CCTCATATTT GGGGCCTTCG 720

CGGTGGGATT AAAAGCAGCG AACCTCGAGA CCAACGTGGA GGAGCTGTGG GTGGAAGTTG 780

GAGGACGAGT AAGTCGTGAA TTAAATTATA CTCGCCAGAA GATTGGAGAA GAGGCTATGT 840

TTAATCCTCA ACTCATGATA CAGACCCCTA AAGAAGAAGG TGCTAATGTC CTGACCACAG 900

AAGCGCTCCT ACAACACCTG GACTCGGCAC TCCAGGCCAG CCGTGTCCAT GTATACATGT 960

ACAACAGGCA GTGGAAATTG GAACATTTGT GTTACAAATC AGGAGAGCTT ATCACAGAAA 1020

CAGGTTACAT GGATCAGATA ATAGAATATC TTTACCCTTG TTTGATTATT ACACCTTTGG 1080

ACTGCTTCTG GGAAGGGGCG AAATTACAGT CTGGGACAGC ATACCTCCTA GGTAAACCTC 1140

CTTTGCGGTG GACAAACTTC GACCCTTTGG AATTCCTGGA AGAGTTAAAG AAAATAAACT 1200

ATCAAGTGGA CAGCTGGGAG GAAATGCTGA ATAAGGCTGA GGTTGGTCAT GGTTACATGG 1260

ACCGCCCCTG CCTCAATCCG GCCGATCCAG ACTGCCCCGC CACAGCCCCC AACAAAAATT 1320

CAACCAAACC TCTTGATATG GCCCTTGTTT TGAATGGTGG ATGTCATGGC TTATCCAGAA 1380

AGTATATGCA CTGGCAGGAG GAGTTGATTG TGGGTGGCAC AGTCAAGAAC AGCACTGGAA 1440

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ACGAGCACTT CAAGGGGTAC GAGTATGTCT CACACATCAA CTGGAACGAG GACAAAGCGG 1560

CAGCCATCCT GGAGGCCTGG CAGAGGACAT ATGTGGAGGT GGTTCATCAG AGTGTCGCAC 1620

AGAACTCCAC TCAAAAGGTG CTTTCCTTCA CCACCACGAC CCTGGACGAC ATCCTGAAAT 1680

CCTTCTCTGA CGTCAGTGTC ATCCGCGTGG CCAGCGGCTA CTTACTCATG CTCGCCTATG 1740

CCTGTCTAAC CATGCTGCGC TGGGACTGCT CCAAGTCCCA GGGTGCCGTG GGGCTGGCTG 1800

GCCTCCTGCT GGTTGCACTG TCAGTGGCTG CAGGACTGGG CCTGTGCTCA TTGATCGGAA 1860

TTTCCTTTAA CGCTGCAACA ACTCAGGTTT TGCCATTTCT CGCTCTTGGT GTTGGTGTGG 1920

ATGATGTTTT TCTTCTGGCC CACGCCTTCA GTGAAACAGG ACAGAATAAA AGAATCCCTT 1980

TTGAGGACAG GACCGGGGAG TGCCTGAAGC GCACAGGAGC CAGCGTGGCC CTCACGTCCA 2040

TCAGCAATGT CACAGCCTTC TTCATGGCCG CGTTAATCCC AATTCCCGCT CTGCGGGCGT 2100

TCTCCCTCCA GGCAGCGGTA GTAGTGGTGT TCAATTTTGC CATGGTTCTG CTCATTTTTC 2160

CTGCAATTCT CAGCATGGAT TTATATCGAC GCGAGGACAG GAGACTGGAT ATTTTCTGCT 2220

GTTTTACAAG CCCCTGCGTC AGCAGAGTGA TTCAGGTTGA ACCTCAGGCC TACACCGACA 2280

CACACGACAA TACCCGCTAC AGCCCCCCAC CTCCCTACAG CAGCCACAGC TTTGCCCATG 2340

AAACGCAGAT TACCATGCAG TCCACTGTCC AGCTCCGCAC GGAGTACGAC CCCCACACGC 2400

ACGTGTACTA CACCACCGCT GAGCCGCGCT CCGAGATCTC TGTGCAGCCC GTCACCGTGA 2460

CACAGGACAC CCTCAGCTGC CAGAGCCCAG AGAGCACCAG CTCCACAAGG GACCTGCTCT 2520

CCCAGTTCTC CGACTCCAGC CTCCACTGCC TCGAGCCCCC CTGTACGAAG TGGACACTCT 2580

CATCTTTTGC TGAGAAGCAC TATGCTCCTT TCCTCTTGAA ACCAAAAGCC AAGGTAGTGG 2640

TGATCTTCCT TTTTCTGGGC TTGCTGGGGG TCAGCCTTTA TGGCACCACC CGAGTGAGAG 2700

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CGAATATCCA GCACTTACTT TACGACCTAC ACAGGAGTTT CAGTAACGTG AAGTATGTCA 2880

TGTTGGAAGA AAACAAACAG CTTCCCAAAA TGTGGCTGCA CTACTTCAGA GACTGGCTTC 2940

AGTGACTTCA GGATGCATTT GACAGTGACT GGGAAACCGG GAAAATCATG CCAAACAATT 3000

ACAAGAATGG ATCAGACGAT GGAGTCCTTG CCTACAAACT CCTGGTGCAA ACCGGCAGCC 3060

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GCGATAAGCC CATCGACATC AGCCAGTTGA CTAAACAGCG TCTGGTGGAT GCAGATGGCA 3120

TCATTAATCC CAGCGCTTTC TACATCTACC TGACGGCTTG GGTCAGCAAC GACCCCGTCG 3180

CGTATGCTGC CTCCCAGGCC AACATCCGGC CACACCGACC AGAATGGGTC CACGACAAAG 3240

CCGACTACAT GCCTGAAACA AGGCTGAGAA TCCCGGCAGC AGAGCCCATC GAGTATGCCC 3300

AGTTCCCTTT CTACCTCAAC GGGTTGCGGG ACACCTCAGA CTTTGTGGAG GCAATTGAAA 3360

AAGTAAGGAC CATCTGCAGC AACTATACGA GCCTGGGGCT GTCCAGTTAC CCCAACGGCT 3420

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TCATCGGAAT CAAGCTCAGT GCCGTGCCCG TGGTCATCCT GATCGCTTCT GTTGGCATAG 3660

GAGTGGAGTT CACCGTTCAC GTTGCTTTGG CCTTTCTGAC GGCCATCGGC GACAAGAACC 3720

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TGCCCACACC CTCCCCTGAG CCACCCCCCA GCGTGGTCCG CTTCGCCATG CCGCCCGGCC 4020

ACACGCACAG CGGGTCTGAT TCCTCCGACT CGGAGTATAG TTCCCAGACG ACAGTGTCAG 4080

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CACCCCTCTA CAGACCGCGC AGAGACGCTT TTGAAATTTC TACTGAAGGG CATTCTGGCC 4380

CTAGCAATAG GGCCCGCTGG GGCCCTCGCG GGGCCCGTTC TCACAACCCT CGGAACCCAG 4440

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GAAGAGAACT GGTTGGAGTT ATGGAAAAGA TGCCCTGTGC CAGGACAGCA GTTCATTGTT 4860

ACTGTAACCG ATTGTATTAT TTTGTTAAAT ATTTCTATAA ATATTTAAGA GATGTACACA 4920

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TGTGTAATAT AGGAAGGAAG GATGTAAAGT GGTATGATCT GGGGCTTCTC CACTCCTGCC 4980

CCAGAGTGTG GAGGCCACAG TGGGGCCTCT CCGTATTTGT GCATTGGGCT CCGTGCCACA 5040

ACCAAGCTTC ATTAGTCTTA AATTTCAGCA TATGTTGCTG CTGCTTAAAT ATTGTATAAT 5100

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TGTTTAAAAT ATTTTAAATT TGCATATCAC AACCCTGTGG TAGTATGAAA TGTTACTGTT 5220

AACTTTCAAA CACGCTATGC GTGATAATTT TTTTGTTTAA TGAGCAGATA TGAAGAAAGC 5280

CCGGAATT 5288

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1447 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```
Met Ala Ser Ala Gly Asn Ala Ala Glu Pro Gln Asp Arg Gly Gly Gly
1               5                  10                  15

Gly Ser Gly Cys Ile Gly Ala Pro Gly Arg Pro Ala Gly Gly Gly Arg
            20                  25                  30

Arg Arg Arg Thr Gly Gly Leu Arg Arg Ala Ala Ala Pro Asp Arg Asp
        35                  40                  45

Tyr Leu His Arg Pro Ser Tyr Cys Asp Ala Ala Phe Ala Leu Glu Gln
    50                  55                  60

Ile Ser Lys Gly Lys Ala Thr Gly Arg Lys Ala Pro Leu Trp Leu Arg
65                  70                  75                  80

Ala Lys Phe Gln Arg Leu Leu Phe Lys Leu Gly Cys Tyr Ile Gln Lys
                85                  90                  95

Asn Cys Gly Lys Phe Leu Val Val Gly Leu Leu Ile Phe Gly Ala Phe
            100                 105                 110

Ala Val Gly Leu Lys Ala Ala Asn Leu Glu Thr Asn Val Glu Glu Leu
        115                 120                 125

Trp Val Glu Val Gly Gly Arg Val Ser Arg Glu Leu Asn Tyr Thr Arg
    130                 135                 140

Gln Lys Ile Gly Glu Glu Ala Met Phe Asn Pro Gln Leu Met Ile Gln
145                 150                 155                 160

Thr Pro Lys Glu Glu Gly Ala Asn Val Leu Thr Thr Glu Ala Leu Leu
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Gln His Leu Asp Ser Ala Leu Gln Ala Ser Arg Val His Val Tyr Met
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SUBSTITUTE SHEET (RULE 26)

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Thr Asn Phe Asp Pro Leu Glu Phe Leu Glu Glu Leu Lys Lys Ile Asn
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His Gly Tyr Met Asp Arg Pro Cys Leu Asn Pro Ala Asp Pro Asp Cys
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Pro Ala Thr Ala Pro Asn Lys Asn Ser Thr Lys Pro Leu Asp Met Ala
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Leu Val Leu Asn Gly Gly Cys His Gly Leu Ser Arg Lys Tyr Met His
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Trp Gln Glu Glu Leu Ile Val Gly Gly Thr Val Lys Asn Ser Thr Gly
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Lys Leu Val Ser Ala His Ala Leu Gln Thr Met Phe Gln Leu Met Thr
        355                 360                 365

Pro Lys Gln Met Tyr Glu His Phe Lys Gly Tyr Glu Tyr Val Ser His
    370                 375                 380

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385                 390                 395                 400

Arg Thr Tyr Val Glu Val Val His Gln Ser Val Ala Gln Asn Ser Thr
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Gln Lys Val Leu Ser Phe Thr Thr Thr Thr Leu Asp Asp Ile Leu Lys
            420                 425                 430

Ser Phe Ser Asp Val Ser Val Ile Arg Val Ala Ser Gly Tyr Leu Leu
        435                 440                 445

Met Leu Ala Tyr Ala Cys Leu Thr Met Leu Arg Trp Asp Cys Ser Lys
    450                 455                 460

Ser Gln Gly Ala Val Gly Leu Ala Gly Val Leu Leu Val Ala Leu Ser
465                 470                 475                 480

Val Ala Ala Gly Leu Gly Leu Cys Ser Leu Ile Gly Ile Ser Phe Asn
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Ala Ala Thr Thr Gln Val Leu Pro Phe Leu Ala Leu Gly Val Gly Val
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Asp Asp Val Phe Leu Leu Ala His Ala Phe Ser Glu Thr Gly Gln Asn
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Lys Arg Ile Pro Phe Glu Asp Arg Thr Gly Glu Cys Leu Lys Arg Thr
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Met Ala Ala Leu Ile Pro Ile Pro Ala Leu Arg Ala Phe Ser Leu Gln
                565                 570                 575

Ala Ala Val Val Val Val Phe Asn Phe Ala Met Val Leu Leu Ile Phe
            580                 585                 590

Pro Ala Ile Leu Ser Met Asp Leu Tyr Arg Arg Glu Asp Arg Arg Leu
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Asp Ile Phe Cys Cys Phe Thr Ser Pro Cys Val Ser Arg Val Ile Gln
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Val Glu Pro Gln Ala Tyr Thr Asp Thr His Asp Asn Thr Arg Tyr Ser
625                 630                 635                 640

Pro Pro Pro Pro Tyr Ser Ser His Ser Phe Ala His Glu Thr Gln Ile
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Thr Met Gln Ser Thr Val Gln Leu Arg Thr Glu Tyr Asp Pro His Thr
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His Val Tyr Tyr Thr Thr Ala Glu Pro Arg Ser Glu Ile Ser Val Gln
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Pro Val Thr Val Thr Gln Asp Thr Leu Ser Cys Gln Ser Pro Glu Ser
    690                 695                 700

Thr Ser Ser Thr Arg Asp Leu Leu Ser Gln Phe Ser Asp Ser Ser Leu
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His Cys Leu Glu Pro Pro Cys Thr Lys Trp Thr Leu Ser Ser Phe Ala
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Glu Lys His Tyr Ala Pro Phe Leu Leu Lys Pro Lys Ala Lys Val Val
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Val Ile Phe Leu Phe Leu Gly Leu Leu Gly Val Ser Leu Tyr Gly Thr
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Thr Arg Val Arg Asp Gly Leu Asp Leu Thr Asp Ile Val Pro Arg Glu
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Thr Arg Glu Tyr Asp Phe Ile Ala Ala Gln Phe Lys Tyr Phe Ser Phe
785                 790                 795                 800

Tyr Asn Met Tyr Ile Val Thr Gln Lys Ala Asp Tyr Pro Asn Ile Gln
                805                 810                 815

His Leu Leu Tyr Asp Leu His Arg Ser Phe Ser Asn Val Lys Tyr Val
            820                 825                 830

Met Leu Glu Glu Asn Lys Gln Leu Pro Lys Met Trp Leu His Tyr Phe
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Arg Asp Trp Leu Gln Gly Leu Gln Asp Ala Phe Asp Ser Asp Trp Glu
    850                 855                 860
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Docket No. 3000.1.0

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Val Leu Ala Tyr Lys Leu Leu Val Gln Thr Gly Ser Arg Asp Lys Pro
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Ile Asp Ile Ser Gln Leu Thr Lys Gln Arg Leu Val Asp Ala Asp Gly
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Ile Ile Asn Pro Ser Ala Phe Tyr Ile Tyr Leu Thr Ala Trp Val Ser
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Asn Asp Pro Val Ala Tyr Ala Ala Ser Gln Ala Asn Ile Arg Pro His
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Arg Pro Glu Trp Val His Asp Lys Ala Asp Tyr Met Pro Glu Thr Arg
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Leu Arg Ile Pro Ala Ala Glu Pro Ile Glu Tyr Ala Gln Phe Pro Phe
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Tyr Leu Asn Gly Leu Arg Asp Thr Ser Asp Phe Val Glu Ala Ile Glu
            980                 985                 990

Lys Val Arg Thr Ile Cys Ser Asn Tyr Thr Ser Leu Gly Leu Ser Ser
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Tyr Pro Asn Gly Tyr Pro Phe Leu Phe Trp Glu Gln Tyr Ile Gly Leu
    1010                1015                1020

Arg His Trp Leu Leu Leu Phe Ile Ser Val Val Leu Ala Cys Thr Phe
1025                1030                1035                1040

Leu Val Cys Ala Val Phe Leu Leu Asn Pro Trp Thr Ala Gly Ile Ile
                1045                1050                1055

Val Met Val Leu Ala Leu Met Thr Val Glu Leu Phe Gly Met Met Gly
            1060                1065                1070

Leu Ile Gly Ile Lys Leu Ser Ala Val Pro Val Val Ile Leu Ile Ala
        1075                1080                1085

Ser Val Gly Ile Gly Val Glu Phe Thr Val His Val Ala Leu Ala Phe
    1090                1095                1100

Leu Thr Ala Ile Gly Asp Lys Asn Arg Arg Ala Val Leu Ala Leu Glu
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His Met Phe Ala Pro Val Leu Asp Gly Ala Val Ser Thr Leu Leu Gly
                1125                1130                1135

Val Leu Met Leu Ala Gly Ser Glu Phe Asp Phe Ile Val Arg Tyr Phe
            1140                1145                1150

Phe Ala Val Leu Ala Ile Leu Thr Ile Leu Gly Val Leu Asn Gly Leu
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Val Leu Leu Pro Val Leu Leu Ser Phe Phe Gly Pro Tyr Pro Glu Val
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Docket No. 50P5150.01

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Gly Pro Ala His Gln Val Ile Val Glu Ala Thr Glu Asn Pro Val Phe
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Asn Pro Arg Gln Gln Pro His Leu Asp Ser Gly Ser Leu Pro Pro Gly
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Arg Gln Gly Gln Gln Pro Arg Arg Asp Pro Pro Arg Glu Gly Leu Trp
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Pro Pro Leu Tyr Arg Pro Arg Arg Asp Ala Phe Glu Ile Ser Thr Glu
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Gly His Ser Gly Pro Ser Asn Arg Ala Arg Trp Gly Pro Arg Gly Ala
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Val Thr Val Ala Val His Pro Pro Pro Val Pro Gly Pro Gly Arg Asn
            1380                1385                1390

Pro Arg Gly Gly Leu Cys Pro Gly Tyr Pro Glu Thr Asp His Gly Leu
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Phe Glu Asp Pro His Val Pro Phe His Val Arg Cys Glu Arg Arg Asp
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Ser Lys Val Glu Val Ile Glu Leu Gln Asp Val Glu Cys Glu Glu Arg
1425                1430                1435                1440

Pro Arg Gly Ser Ser Ser Asn
                1445
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09/534,150 - 03/24/00

WHAT IS CLAIMED IS:

1. An assay for phenotyping the *patched* status of a cell, comprising detecting, in a sample of mammalian cells, the presence or absence of a genetic lesion characterized by at least one of (i) aberrant modification or mutation of a *patched* gene, and (ii) mis-expression of said *patched* gene.

2. The assay of claim 1, wherein detecting said lesion includes:
   i. providing a diagonistic probe comprising a nucleic acid including a region of nucleotide sequence which hybridizes to a sense or antisense sequence of said *patched* gene, or naturally occuring mutants thereof, or 5' or 3' flanking sequences naturally associated with said gene;
   ii. combining said probe with nucleic acid from said cell sample; and
   iii. detecting, by hybridization of said probe to said cellular nucleic acid, the existence of at least one of a deletion of one or more nucleotides from said *patched* gene, an addition of one or more nucleotides to said *patched* gene, a substitution of one or more nucleotides of said *patched* gene, a gross chromosomal rearrangement of all or a portion of said *patched* gene, a gross alteration in the level of an mRNA transcript of said *patched* gene, or a non-wild type splicing pattern of an mRNA transcript of said *patched* gene.

3. The assay of claim 2, wherein hybridization of said probe further comprises subjecting the probe and cellular nucleic acid to a polymerase chain reaction (PCR) and detecting abnormalities in an amplified product.

4. The assay of claim 2, wherein said probe hybridizes under stringent conditions to a nucleic acid designated by SEQ ID No. 9 or 18.

5. The assay of claim 2, wherein said probe hybridizes under stringent conditions to a nucleic acid designated by SEQ ID No. 18.

6. The assay of claim 2, wherein said probe further comprises a label group attached to said nucleic acid and able to be detected.

7. The assay of claim 1, wherein detecting said lesion comprises ascertaining, from a methylation pattern of said *patched* gene, the presence or absence of aberrant methylation of said *patched* gene.

8. The assay of claim 7, wherein the methylation pattern of said *patched* gene is determined by combining nucleic acid of said cell sample with one or more methylation-sensitive restriction endonucleases and determining the restriction digest pattern of at least a portion of said *patched* gene.

9. The assay of claim 1, wherein detecting said lesion comprises detecting the presence or absence of a non-wild type level of a *patched* protein product of said *patched* gene in cells of said cell sample.

10. The assay of claim 9, wherein the level of said *patched* protein is detected in an immunoassay.

11. The assay claim 1, wherein detecting said lesion comprises ascertaining, relative to a wild-type level of *hedgehog*-dependent *patched* signal transduction, the ability of cells in said cell sample to respond to *hedgehog* induction.

12. The assay of claim 1, wherein said cell sample is obtained from a human patient.

13. A method for diagnosing a genetic predisposition of an animal for at least one of a developmental abnormality or a proliferative disorder marked by abberant expression or activity of a *patched* gene or gene product, the method comprising detecting the presence of a predisposing mutation in a *patched* gene in cells of said animal, wherein the presence of said predisposing mutation indicates that said individual has a genetic predisposition for at least one of developmental abnormalities or a proliferative disorder.

14. The method of claim 13, wherein said genetic predisposition is basal cell nevus syndrome.

15. The method of claim 13, wherein said genetic predisposition is a predisposition for developing a carcinoma

16. The method of claim 13, wherein said genetic predisposition is a predisposition for developing a meningiomas.

17. The method of claim 13, wherein said genetic predisposition is a predisposition for developing a medullomas

18. The method of claim 13, wherein said genetic predisposition is a predisposition for developing a fibroma.

19. The method of claim 13, wherein said detecting step comprises analyzing a nucleic acid sample obtained from said animal.

20. The method of claim 13, wherein said detecting step comprises functional analysis of *patched* protein function.

21. The method of claim 13, wherein said detecting step comprises detecting antibody binding to abnormal *patched* protein.

22. A method for characterizing the phenotype of a tumor, comprising detecting the presence of an oncogenic *patched* mutation in cells of the tumor, wherein the presence

of said oncogenic mutation indicates that said tumor has a patched-associated phenotype.

20. The method of claim 19, wherein said tumor is a carcinoma.

21. The method of claim 20, wherein said carcinoma is a basal cell carcinoma.

22. The method of claim 19, wherein said tumor is a meningioma.

23. The method of claim 19, wherein said tumor is a medulloma

24. The method of claim 19, wherein said tumor is a fibroma.

25. The method of claim 19, wherein said oncogenic *patched* mutation are detected by analyzing DNA of said tumor.

26. The method of claim 19, wherein said oncogenic *patched* mutation are detected by mRNA of said tumor.

27. The method of claim 19, wherein said detecting step comprises functional analysis of patched protein function.

28. The method of claim 19, wherein said detecting step comprises detecting antibody binding to abnormal patched protein.

29. A genetically engineered mammalian cell predisposed to develop a proliferative phenotype as a result of transfection of said mammalian cell with at least one nucleic acid construct which inhibits expression of an endogenous *patched* gene or alters the signal transduction activity of a wild-type *patched* protein.

30. The cell of claim 26, wherein the cell develops a carcinoma phenotype.

31. The cell of claim 30, wherein the cell develops a basal cell carcinoma phenotype.

32. The cell of claim 26, wherein the cell develops a meningioma phenotype.

33. The cell of claim 26, wherein the cell develops a medulloma phenotype.

34. The cell of claim 26, wherein the cell develops a fibroma phenotype.

35. A method for treating an animal having a disorder characterized by loss-of-function of a *patched* gene, comprising transfecting cells of the animal with an expression construct encoding a *patched* protein.

36. The method of claim 35, wherein the cells are transfected *in vivo*.

37. The method of claim 35, wherein the cells are transfected *in vitro*.

38. The method of claim 35, wherein the expression construct is a viral vector.

39. The method of claim 35, wherein the transfected cells include epithelial cells.

40. The method of claim 35, wherein the transfected cells include neuronal cells.

41. The method of claim 35, wherein the transfected cells include carcinoma cells.

42. The method of claim 41, wherein the carcinoma cells are basal cell carcinoma cells.

43. The method of claim 35, wherein the transfected cells include meningioma cells.

43. The method of claim 35, wherein the transfected cells include medulloma cells.

44. The method of claim 35, wherein the transfected cells include fibroma cells.

45. A method for treating an animal having a disorder characterized by loss-of-function of a *patched* gene, comprising administering to the animal an agent which inhibits derepression of one or more *patched*-dependent genes.

Document No. 3 Received on 05/15/2001

PATCHED GENES AND THEIR USES

ABSTRACT OF THE INVENTION

Methods for isolating patched genes, particularly mammalian patched genes, including the mouse and human *patched* genes, as well as invertebrate patched genes and sequences, are provided. Decreased expression of *patched* is associated with the occurrence of human cancers, particularly basal cell carcinomas of the skin. The cancers may be familial, having as a component of risk an inherited genetic predisposition, or may be sporadic. The *patched and hedgehog* genes are useful in creating transgenic animal models for these human cancers. The *patched* nucleic acid compositions find use in identifying homologous or related proteins and the DNA sequences encoding such proteins; in producing compositions that modulate the expression or function of the protein; and in studying associated 15 physiological pathways. In addition, modulation of the gene activity *in vivo* is used for prophylactic and therapeutic purposes, such as treatment of cancer, identification of cell type based on expression, and the like. The DNA is further used as a diagnostic for a genetic predisposition to cancer, and to identify specific cancers having mutations in this gene.

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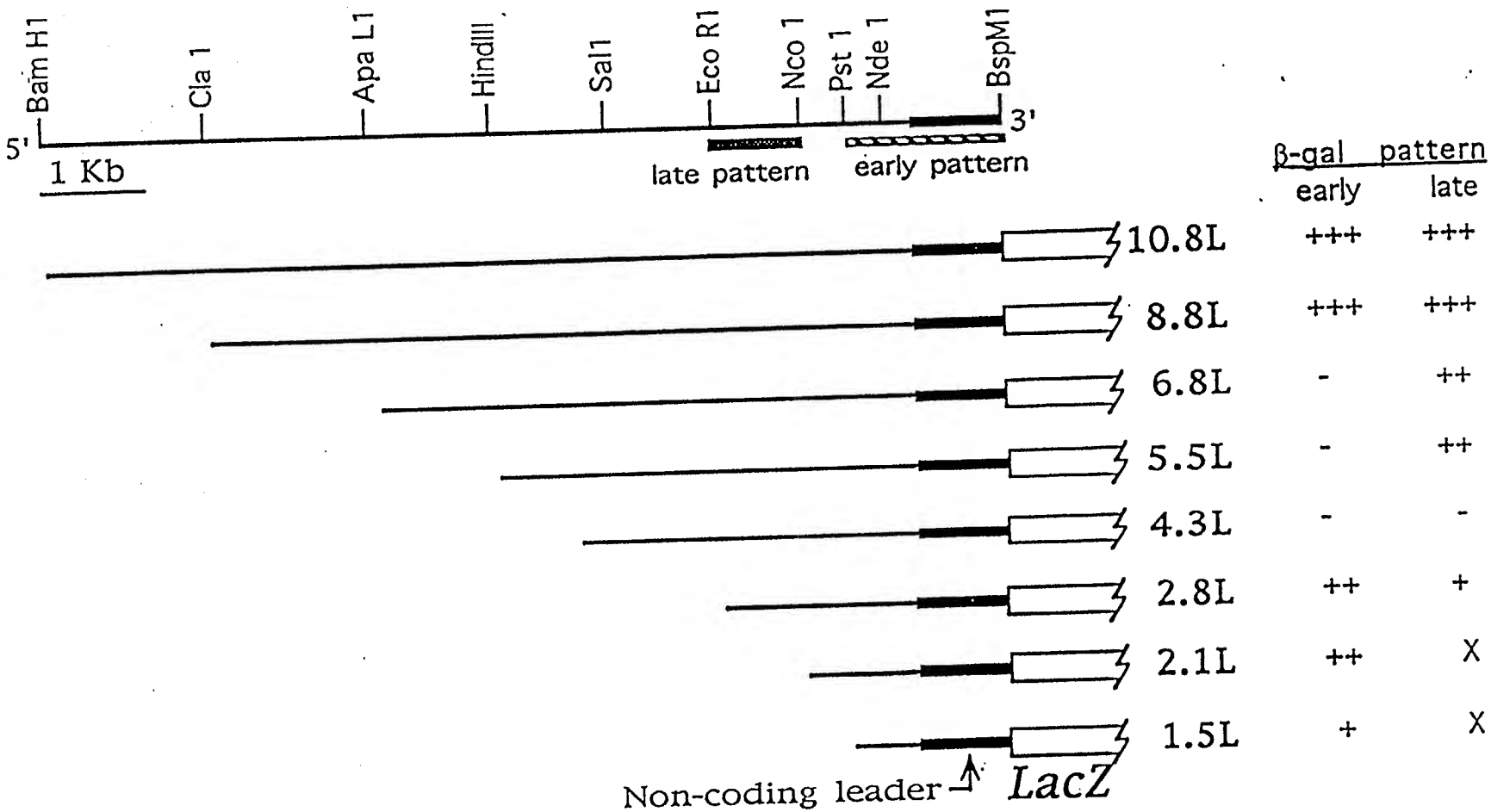

FIGURE 1

FIGURE 2

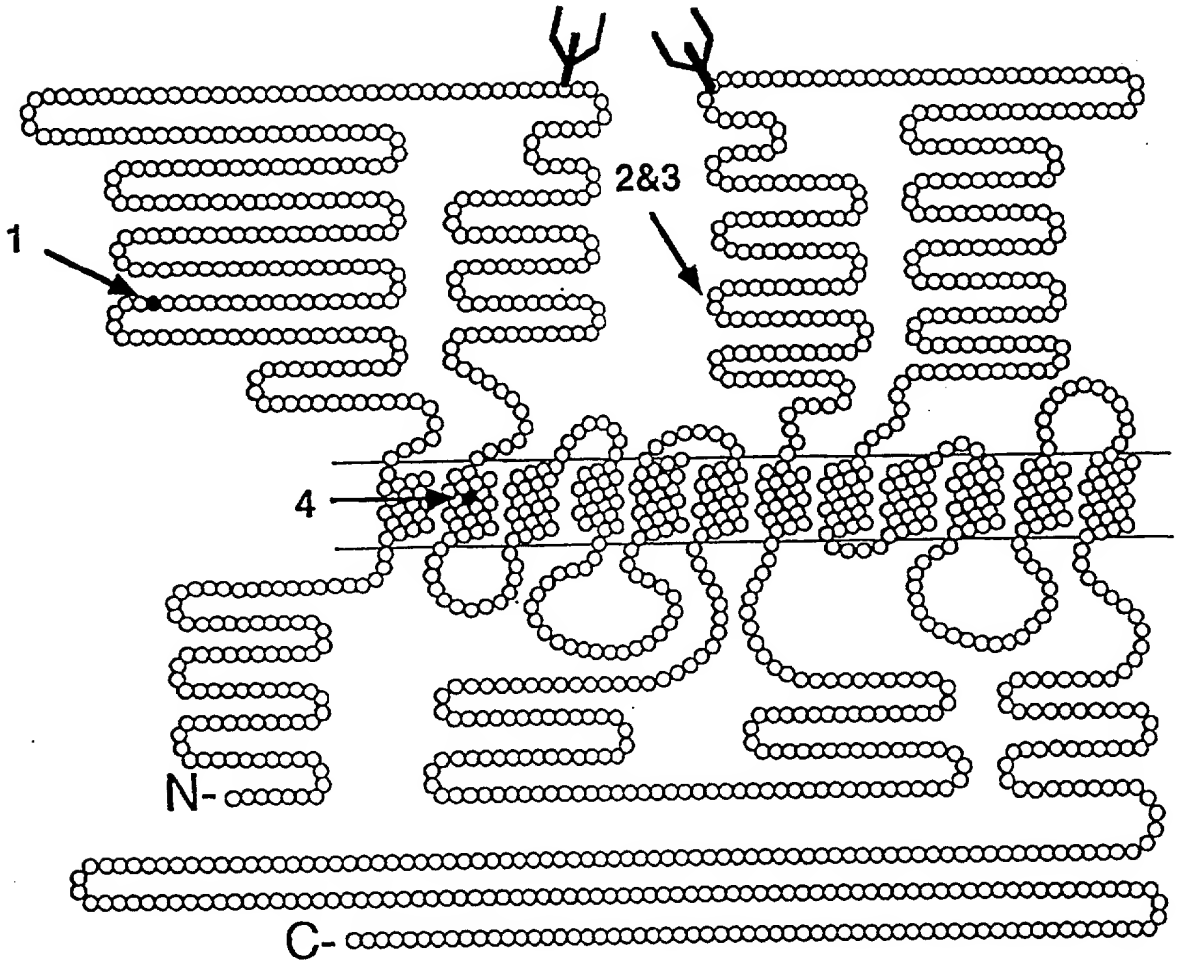

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Figure 3

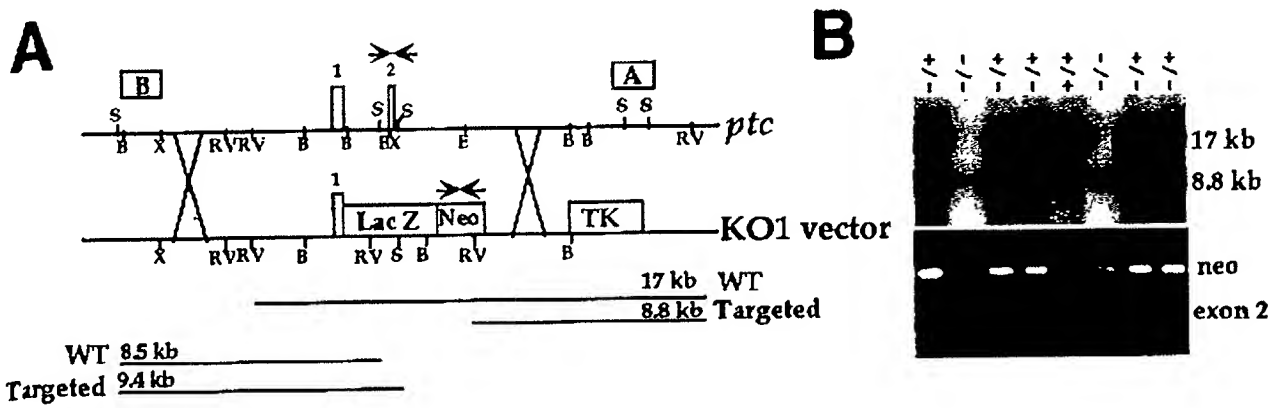
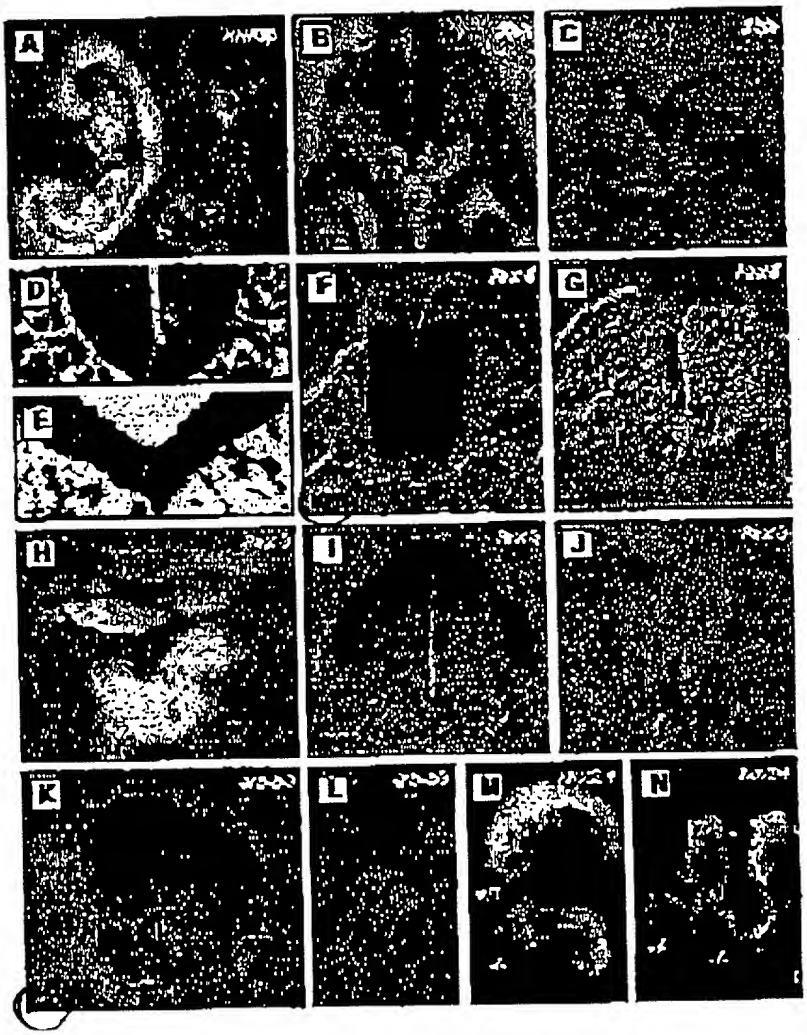


Figure 5



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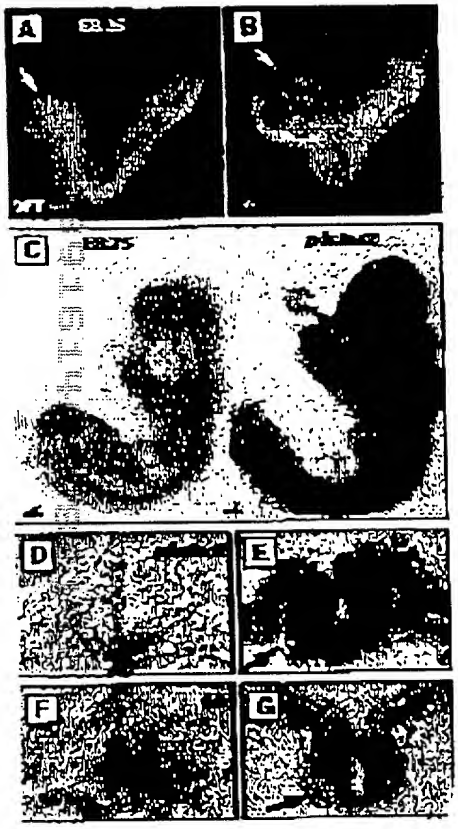

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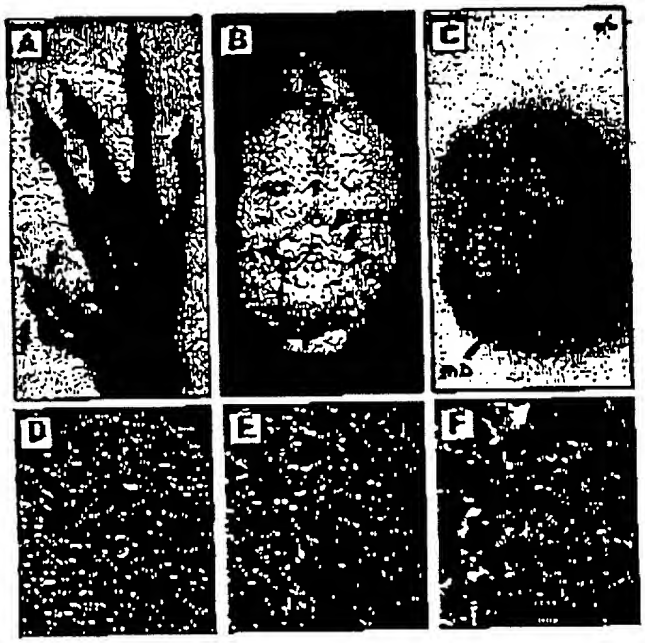

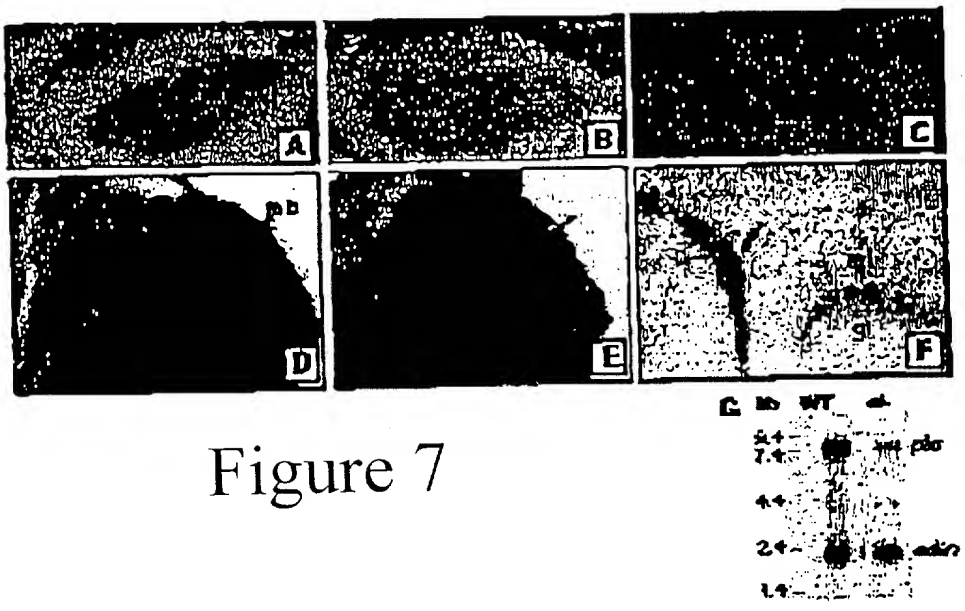

Figure 7

Attorney's Docket Number: SUV-003.04

Declaration, Petition and Power of Attorney For Patent Application

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled:

PATCHED GENES AND USES RELATED THERETO

the specification of which is filed herewith in the U.S. Patent and Trademark Office.

I do not know and do not believe that the subject matter of this application was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to the date of this application, and that said subject matter has not been patented or made the subject of an issued inventor's certificate in any country foreign to the United States on an application filed by me or my legal representatives or assigns more than twelve months prior to the date of this application; that I acknowledge my duty to disclose information of which I am aware which is material to the examination of this application, that no application for patent or inventor's certificate on the subject matter of this application has been filed by me or my representatives or assigns in any country foreign to the United States, except those identified below, and that I have reviewed and understand the contents of the specification, including the claims as amended by any amendment referred to herein.

I acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, §1.56(a).

PRIORITY CLAIM

I hereby claim foreign priority benefits under Title 35, United States Code, §119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

Check one: X no such applications have been filed.
__ such applications have been filed as follows

EARLIEST FOREIGN APPLICATION(S), IF ANY FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

Country	Application Number	Date of Filing (month,day,year)	Priority Claimed Under 35 USC 119
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No _
			_ Yes No

(6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

CLAIM FOR BENEFIT OF EARLIER U.S./PCT APPLICATION(S)

I hereby claim the benefit under Title 35, United States Code, §120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, §112, I acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, §1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application. As to subject matter of this application which is common to my earlier United States application, if any, described below, I do not believe that the same was ever known or used in the United States before my invention thereof or patented or described in any printed publication in any country before my invention thereof or more than one year prior to said earlier application, or in public use or on sale in the United States more than one year prior to said earlier application, that the said common subject matter has not been patented or made the subject of an inventor's certificate issued before the date of said earlier application in any country foreign to the United States on an application, filed by me or my legal representatives or assigns more than twelve months prior to said application and that no application for patent or inventor's certificate on said subject matter has been filed by me or my representatives or assigns in any country foreign to the United States except those identified herein.

08/656,055	31 May 1996	PENDING
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)
08/540,406	06 October 1995	PENDING
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)
08/319,745	07 October 1994	ABANDONED
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, aband.)

DOCKET NO. 2000.1

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorneys and/or agents to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

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Matthew P. Vincent	Reg. No. 36,709	Edward J. Kelly	Reg. No. 38,936
		Sayoko Blodgett-Ford	Reg. No. 40,516

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Direct Telephone Calls to: Matthew P. Vincent, Ph.D. (617) 832-1000

Wherefore I petition that letters patent be granted to me for the invention or discovery described and claimed in the attached specification and claims, and hereby subscribe my name to said specification and claims and to the foregoing declaration, power of attorney, and this petition.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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DOCKET NO. 20-5